27 The method of Claim 21, wherein said contacting step comprises administering said self antigen preparation and said CTLA-4 blocking agent to said mammalian host simultaneously or sequentially.

31. The method of Claim 21, comprising contacting said mammalian T cell with an immune response stimulating agent either simultaneously or sequentially.

**REMARKS**

Claims 22, 29, 30 & 32 have been cancelled without prejudice. After entry of the amendments, claims 21, 23-28 and 31 remain pending in the application. Claims 21, 23-26 and 31 have been amended. Support is found in the specification on pages 19-20 and in Examples 10-12 at pages 47-67. The Examiner objects to claim 30 as being improperly dependent on itself. Claim 30 has been cancelled, thus obviating the objection.

No new matter is added by the amendments. Favorable consideration of the following comments relative to the outstanding rejections as they may apply to the present claims is respectfully requested for the reasons that follow.

**Summary of the Invention**

By way of summary, the pending claims are drawn to methods of inhibiting the growth of non-T cell tumors through the use of a CTLA-4 blocking agent and a self antigen preparation, wherein the combination is effective in breaking immune tolerance to the self antigen and stimulating an autoreactive T cell response. The CTLA-4 blocking agent is recited as having the following two characteristics: 1) it binds to the extracellular domain of the CTLA-4 receptor; and 2) it is inhibitory of CTLA-4 mediated signaling. A "self antigen" is expressly defined in the specification as being an antigen which is present in both normal cells and tumor cells, or alternatively a normal gene product with highly restricted cellular distribution, to which a host will have natural immune tolerance (page 2, lines 20-22; page

19, lines 11-13). Following the methods of the present invention, one may effectively inhibit the growth of non-T cell tumors, and non-immunogenic or poorly immunogenic tumors in particular, by abrogating the host's natural immune tolerance to such a self antigen (page 2, lines 17-24, Examples 10-12 at pages 47-67).

As explained in the specification, stimulating low-avidity autoreactive T-cells or naive T cells against a self antigen provides a cytotoxic response against non-T cell tumors through recognition of self antigens on the tumor cell (page 2, lines 17-29; page 3, lines 20-27; page 5, lines 17-20; page 19, lines 9-17). This is demonstrated for the first time in Examples 10 & 11 at pages 47-66, wherein the combination of a CTLA-4 blocking agent and a self antigen preparation resulted in inhibition of tumor cell growth and an autoreactive T cell response against normal cells bearing the tissue-specific self-antigen. This approach provides an alternative way of inhibiting tumor growth in addition to any cytotoxic response that may be mediated through immune response directed against tumor antigens, which are present in the tumor cell in an abnormal context, are uniquely expressed by the tumor cell, or are a mutated form of a normal, unaltered self-antigen (page 17, lines 25-27).

**Rejections Under 35 U.S.C. § 112, first paragraph: Enablement**

Claims 21-25, 27-29 and 31 stand rejected under 35 U.S.C. § 112, first paragraph for lack of enablement. Generally, the Examiner contends the claimed compositions, while enabled for inhibiting non-T cell tumors in mouse with CTLA-4 blocking agent comprising 9H10 antibody and immune stimulating agent comprising GM-CSF producing tumor cells or GM-containing microspheres, are not enabled for inhibiting the growth of "any non-T cell tumor cells" in "any mammal" using "any CTLA-4 blocking agent" commensurate with the scope of the claims. A number of reasons are cited in holding the compositions nonenabling.

First, the Examiner contends that making any of the claimed CTLA-4 blocking agents requires undue experimentation, notwithstanding the exemplary blocking agent 9H10 described in the specification. Second, the Examiner argues that immune response

stimulating agents are nonenabled because of a lack of guidance in the specification regarding making and using the stimulating agents, the ineffectiveness of the stimulating agents as antigens, and the potential harm to patients treated with stimulating agents. Third, the Examiner concludes that even if CTLA-4 blocking agents and immune response stimulating agents are enabled, the claimed compositions are not enabled for inhibiting growth of any tumor because of the unpredictable effectiveness of cancer vaccines and uncertainties regarding the immune response elicited by the claimed methods. Fourth, the Examiner regards the claimed methods ineffectual in view of the ineffectiveness of other cancer vaccines. Fifth, the methods are nonenabled in view of the Examiner's apparent doubts about the credibility of CTLA-4 blocking agents to inhibit CTLA-4 mediated signaling, the alleged lack of tumor growth inhibiting activity at late stages of tumor progression, the potential harm to patients treated with the claimed method, and the potential elimination of tumor cell responsive T-cells by CTLA-4 blockade. Finally, since the claimed methods are allegedly not enabled for treating tumors of any kind, the Examiner considers the claimed methods are not enabled for treating any mammal, especially for therapy directed to human cancer patients in view of a lack of guidance regarding preparing and administering the compositions of the method in a form suitable for use in a human clinical setting.

As explained in detail below, Applicants respectfully traverse the Examiner's enablement rejection for the following reasons: 1) the disclosure in the instant specification together with the knowledge of the skilled artisan at the time the invention was made is more than adequate to enable the claimed CTLA-4 blocking agents and self-antigen preparations; 2) the numerous tumor vaccine references cited against the enablement of the claimed method are largely inapposite in that they do not address the use of a CTLA-4 blocking agent in combination with such a vaccine, and thus they are inadequate to call into question the enablement of Applicants' properly exemplified method, and 3) the additional references relating to proposed mechanisms of CTLA-4 action are improperly cited as implicating the presently-claimed method. Furthermore, although Applicants object to the Examiner's

arguments relating to safety and efficacy in treating human cancer as not required for patentability, Applicants further note that the development of CTLA-4-based cancer strategies has, in fact, progressed to successful Phase I human clinical trials in prostate cancer and melanoma.

Thus, as indicated herein, Applicants submit that the claimed method of inhibiting the growth of non-T cell tumors using a CTLA-4 blocking agent and a self antigen preparation to break immune tolerance and stimulate an autoreactive T cell response against the self antigen is fully enabled.

*Enablement of CTLA-4 Blocking Agents*

The Examiner considers the claimed methods nonenabled because undue experimentation is required to make "any" CTLA-4 blocking agent. The Examiner dismisses the exemplified 9H10 monoclonal antibody as failing to define other CTLA-4 blocking agents, including other antibodies, since the disclosure does not expressly describe these other blocking agents. Moreover, other CTLA-4 blocking antibodies are found nonenabled because of what the Examiner considers the unpredictable effect of any antibody directed against a cell surface receptor, even if the antibody inhibits CTLA-4 mediated signaling. Consequently, the Examiner reaches the conclusion that no other CTLA-4 blocking agent is enabled. Applicants respectfully disagree.

Applicants respectfully remind the Examiner that sufficiency of enablement is not whether experimentation is necessary to practice the claimed invention, but whether the experimentation is undue. See In re Wands, 858 F.2d 731, 738, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). The Federal Circuit stated that

> [t]he determination of what constitutes undue experimentation in a given case requires the application of a standard of reasonableness . . . . The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed.

In Wands, the claims were drawn to methods of immunoassay of HbsAg by use of high-affinity monoclonal antibodies. The Patent Office and the Board of Patent Appeals rejected the assertion that the claims encompassed all monoclonals having the recited affinity because undue experimentation was needed to make such monoclonal antibodies. The Federal Circuit reversed, holding that the experimentation required was not undue. In its analysis, the Federal Circuit outlined the steps for making monoclonal antibodies:

(a) an animal is immunized with the antigen of choice;
(b) after a period of time, the spleen is removed
(c) the lymphocytes are separated from the other spleen cells;
(d) the lymphocytes are mixed with myeloma cells, and treated to fuse the cells; and
(e) hybridoma antibodies that secrete the desired antibodies must be isolated from the enormous mixture of other cells, using a series of screening steps, including,
    (i) culturing the cells such that only hybridoma cells will grow;
    (ii) hybridomas are isolated and cloned, by placing single hybridoma cells in separate chambers and growing them;
    the secreted antibodies from each clone are screened to see it they bind to the antigen, a step which frequently requires the screening of "hundreds" of clones.

The Court stated that although the amount of experimentation in making the monoclonals was large, the experimentation was merely routine and thus not undue under 35 U.S.C. § 112, first paragraph.

In view of the amount of experimentation required in Wands, the Federal Circuit enumerated that the factors to consider in determining whether experimentation is undue include (a) the nature of the invention, (b) state of the prior art, (c) relative skill of those in the art, (d) the amount of direction in the specification, (e) the presence or absence of working examples, (f) the predictability or unpredictability of the art, (g) breath of the claims, and (h) the quantity of experimentation which would be required in order to practice the invention as claimed. The Applicants submit that the experimentation required to make CTLA-4 blocking agents is routine and not undue.

Regarding the nature of the invention, the claimed CTLA-4 blocking agents are defined as agents which bind to the extracellular domain of CTLA-4 and prevent CTLA-4 mediated signaling that leads to non-responsiveness of T cells. Thus, the nature and function of blocking agents are clearly enumerated as to their effect on the T-cells and CTLA-4 receptor signaling events.

Regarding the state of the prior art, the technology for making and screening antibodies, peptides, and organic compounds with specific properties was well known in the art at the time of filing of the instant application. Making polyclonal and monoclonal antibodies directed against specific peptides was technologically advanced and routine. Furthermore, techniques for making synthetic peptides and small molecule organic compounds were well-developed in their relevant fields.

Regarding the relative skill in the art, Applicants submit that the level of skill in the art is high. The technical expertise in making antibodies, including humanized antibodies, is considerable given the experience in the field. Moreover, the skill in the art of peptide and small molecule chemistry is highly advanced for synthesizing peptidomimetic and organic molecules for use in screening procedures employed to identify agents having defined biological activity.

Regarding the amount of direction given in the specification, the disclosure specifies the necessary properties of the claimed CTLA-4 blocking agent, including its effect on the T cell response, and provides detailed methods for making blocking antibodies and describes the specific screening process for identifying other agents with the desired blocking property (pages 25-32). The specification further describes methods for making humanized antibodies and monoclonal antibodies directed against human CTLA-4 (page 13, lines 23-30, page 14, and page 40-43). Thus, considerable guidance and direction is provided in the disclosure to allow a skilled artisan to use the described assay to identify other blocking agents useful in the claimed compositions.

Regarding the presence or absence of working examples, the specification provides an exemplary blocking agent in the 9H10 monoclonal antibody. The 9H10 binds to CTLA-4 extracellular domain, and when used in a composition comprising the 9H10 antibody and an immune stimulating agent, the composition is effective in inhibiting the growth of various tumors. Moreover, the Federal Circuit has held that evidence of a single monoclonal antibody with specific binding property to a cell surface molecule is sufficient to encompass the genus of monoclonal antibodies with similar activity, even when the patentee subsequently encounters difficulty or uncertainty in making other similar antibodies. See Johns Hopkins Univ. v. Cellpro Inc., 152 F.3d 1342, 47 USPQ2d 1705 (Fed. Cir. 1998).

Regarding the predictability or unpredictability of the art, producing CTLA-4 blocking agents is not an unpredictable art. As regards to antibodies, the Federal Circuit held in Wands that the method for making monoclonal antibodies with specific affinities to an antigen was not unpredictable given the skill and knowledge of those in the art. 8 USPQ2d at 1400. This view was maintained by the Federal Circuit in Johns Hopkins Univ. v. Cellpro even when the patentee could produce only a single monoclonal and others skilled in art had difficulty generating similar antibodies. 47 USPQ2d at 1781. Thus, exemplification of other antibodies is not required to show enablement of the claimed genus of antibodies.

Moreover, Applicants submit that in view of the screening process described in the specification, a person skilled in the art can readily identify other agents (i.e. peptidomimetic or organic molecules) which bind the extracellular domain of CTLA-4 receptor and inhibit CTLA-4 mediated signaling. In support, Applicants direct the Examiner to Exhibit A, Devlin, J.J. "Random peptide libraries: a source of specific protein binding molecules," (1990) Science, 249: 404-6 (hereinafter "Devlin"); Exhibit B, Oldenbrug, K.R. "Peptide ligands for a sugar-binding protein isolated from a random peptide library," (1992) Proc. Natl. Acad. Sci. USA 89: 5393-97 (hereinafter "Oldenburg"); Exhibit C, Sparks, A.B. "Identification and characterization of Src SH3 ligand from phage displayed random peptide libraries," (1994) J. Biol. Chem. 269: 23853-56 (hereinafter "Sparks"); Exhibit D, Goodson,

R.J., "High-affinity urokinase receptor antagonists identified with bacteriophage peptide display," (1994) Proc. Natl. Acad. Sci. USA, 91: 7129-33 (hereinafter "Goodson"); Exhibit E, Yanofsky, S.D., "High affinity type I interleukin receptor antagonists discovered by screening recombinant peptide libraries," (1996) Proc. Natl. Acad. Sci. USA 93: 7381-86 (hereinafter "Yanofsky"); Exhibit F, Chaudhary, J. et al."Caloxin: a novel plasma membrane Ca+2 pump inhibitor;" (2001) Am. J. Physiol. 280 (hereinafter "Chaudhary"): C1027-30; and Exhibit G, Xu, H. et al., "A novel PCNA binding motif identified by the panning of a random peptide display library," (2001) Biochemistry, 40: 4512-20 (hereinafter "Xu"). The foregoing references are offered to show that the specification fully enables the CTLA-4 blocking agents given the considerable guidance given in the specification about the nature of the invention and the described screening methods.

For example, the references of Devlin, Oldenburg, Sparks, and Goodson describe the state of the prior art in synthesizing and screening random peptide libraries, which was well-developed at the time of filing of the instant application. In particular, Goodson show the identification of peptides that selectively bind a cell surface receptor, urokinase. Yanofsky demonstrate that given a specific assay for a binding agent, screening of peptide libraries can lead to agents that selectively bind target cell surface receptors and inhibit signaling events. Furthermore, Chaudhary and Xu demonstrate the applicability of such methods to other proteins if a method for assaying the binding activity is available. As the Federal Circuit stated in Gould v. Quigg, 3 USPQ2d 1302, 1305 (Fed. Cir. 1987)

> it is true that a later dated publication cannot supplement an insufficient disclosure in a prior dated application to render it enabling. In this case the later dated publication was not offered as evidence for this purpose. Rather, it was offered . . . as evidence that the disclosed device would have been operative.

See also In re Wilson, 135 USPQ 442, 444 (CCPA); Ex parte Obukowicz, 27 USPQ2d 1063 (BPAI 1993). Thus, Applicants submit that obtaining CTLA-4 blocking agents with the described properties is not unpredictable and demands just routine experimentation.

Regarding the breadth of the claims, claim 21 specifically recites that the blocking agent is capable of specifically binding to the extracellular domain of CTLA-4 and inhibiting CTLA-4 mediated signaling. Thus, the claimed blocking agents are sufficiently defined to apprise those skilled in the art as to the scope of the claimed subject matter.

Regarding the quantity of experimentation necessary to make the claimed blocking agents, the amount of experimentation is similar if not identical to the screening of monoclonal antibodies at issue in Wands. In the present case, the experimentation will follow essentially the steps outlined in Wands. Animals are immunized with extracellular domain of CTLA-4, or peptide libraries or small organic molecules are synthesized by well-known methods. For monoclonal antibodies, hybridoma cell lines secreting the antibodies to be tested are made by methods described in the specification and by techniques well-known to those skilled in the art. The antibodies, peptide library, or the small molecule library are screened for their ability to bind the extracellular domain of CTLA-4 as provided in Example 1 of the specification. Candidate agents that selectively bind CTLA-4 are characterized by the methods on pages 29-32 and identified according to the set of criteria given in the specification on page 8-9. Thus, similar to Wands and Johns Hopkins Univ., the amount of screening required may be large, but the experimentation required to obtain other CTLA-4 blocking agents is routine and not undue.

In view of the foregoing, Applicants submit that the Examiner has applied a higher standard than required by the Federal Circuit in determining enablement of CTLA-4 blocking agents. Given the considerable direction and guidance provided in the specification and the high level of skill in the art, making and identifying CTLA-4 blocking agents require experimentation which is routine and not undue. Accordingly, Applicants submit that CTLA-4 blocking agents other than the exemplary 9H10 are fully enabled by the specification.

*Enablement of Immune Response Stimulating Agents*

The Examiner contends the claimed methods are nonenabled because the specification does not exemplify use of tumor cells or tumor cell lysates Applicants respectfully traverse.

The Applicants direct the Examiner to M.P.E.P. § 21634.05(a), which states "the specification need not disclose what is well known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public." Thus "[a]n inventor need not . . . explain every detail since he is speaking to those skilled in the art." See DeGeorge v. Bernier, 768 F.2d 1318, 226 USPQ 758, 762 (Fed. Cir. 1985).

The use of tumor cells, tumor cell lysates, and tumor cells transduced with nucleic acids expressing cytokines have been well known to those skilled in the art of cancer immunotherapy, as the literature in the subject area attests. As already pointed out by the Examiner, references cited in the Office Action describe use of tumor lysates to elicit immune response against cancer cells in the early years of the emerging field of cancer immunotherapy (see Bodey, B. et al. (2000) Anticancer Res. 20: 2665-2676) ("Classical and molecular immunological means of active tumor specific immunization against human cancers yielded whole cell or tumor cell lysate vaccines of preventive value . . . .").

Moreover, Applicants submit that the purified self-antigens of claim 26 are well-known to those skilled in the art and were available in their purified form or readily purified by methods known at the time of filing of the instant application. Accordingly, undue experimentation is not required for making and using purified self antigens.

The Examiner, however, asserts other reasons why the immune response against self antigens agents are in general nonenabled. First, the Examiner finds unpredictable whether the self-antigens, the peptide fragments of these self-antigens, or related synthetic peptides will be sufficiently immunogenic to elicit stimulatory effects. Second, under a related issue, the Examiner finds presentation of the antigens at the cell surface in context of MHC class I molecule as inadequate to prove effective for cancer therapy. Third, the Examiner hypothesizes that inadvertent activation of a non-specific immune response will elicit "multi-

focal autoimmune response," which would harm the mammalian host. Applicants respectfully traverse.

In determining enablement, the Federal Circuit emphasizes that the test is one of reasonableness. See M.P.E.P § 2164.01. Thus, the Examiner must provide a reasonable basis to question the enablement of the claimed subject matter and create sufficient reason to doubt the objective truth of the contents of the disclosure. See M.P.E.P § 2164.04.

In the instant case, the Examiner arrives at hypothetical conclusions unsupported by specific findings of fact and fails to advance sufficient reason to show nonenablement of the immune stimulating agents comprising self antigens as recited in claims. The specification describes on page 64 that combined treatment with CTLA-4 blocking agent and prostate cancer cell expressing GM-CSF elicited mononuclear cell infiltration and destruction of glandular epithelium of the male reproductive tract of normal mouse. This result demonstrates an immune response directed against antigens expressed on normal and cancerous prostate cells (i.e. self antigens), as does the vitiligo resulting from treatment of mice with anti-CTLA-4 and BL6/GM described in Example 10. Moreover, Example 12 demonstrates that combination of a CTLA-4 blocking agent and a murine melanocyte-specific protein (gp100) expressed on normal melanocytes results in inhibition of B16 melanoma tumor growth in mice. This result confirms the effectiveness of purified self antigen in breaking self-tolerance when used with CTLA-4 blockade.

Regarding the "potential" for indiscriminate stimulation of T-cells by CTLA-4 blocking agent and the possibility of eliciting harmful autoimmune reactions, the M.P.E.P. instructs that the issue of safety of the claimed subject matter is not the province of the Patent Office and not a requirement for satisfying enablement under 35 U.S.C. § 112, first paragraph. See M.P.E.P § 2164.05. Speculation as to possible adverse effects of a claimed therapeutic protocol are the province of other governmental agencies. Moreover, the specification indicates that "rather than being viewed as a troublesome side effect of tumor immunotherapy, the intentional induction of autoimmunity to defined tissue specific antigens

can provide a practical strategy for the generation of effective anti-tumor responses." (page 66). There are many areas of oncology where complete elimination of the affected tissue would be advantageous. Indeed, that is quite often the focus of surgical resection. In view of the foregoing, Applicants submit the Examiner has not established a reasonable basis to conclude nonenablement of immune response stimulating agents in general or the claimed self antigen preparations under § 112, first paragraph.

*Unpredictability in the Art of Cancer Immunotherapy*

The Examiner appears to hold that even if CTLA-4 blocking agents and immune response stimulating agents are enabled, they are not enabled for inhibiting any tumor growth because of the unpredictability and uncertainties of treating tumors using immunotherapy. Thus, the Examiner appears to assert that any composition comprising a CTLA-4 blocking agent and immune stimulatory agent comprising a self antigen is nonenabling. Applicants respectfully traverse.

The Examiner infers the general unpredictability in the field by citing the teachings of Bodey, B. et al. (2000) Anticancer Res. 20: 2665-2676 (hereinafter "Bodey"), Ezzell, C. (1995) J. NIH Res. 7, 46-49 (hereinafter "Ezzell"), and Spitler, L.E. (1995) Cancer Biotherapy, 10: 1-3 (hereinafter "Spitler").

Bodey summarizes various approaches used to activate the immune response against tumor cells, including use of vaccines such as whole cell tumor lysates, anti-idiotypic antibodies, and tumor associated antigens (TAA). Although the treatments lead to regression of some tumors, they do not always lead to complete elimination. Bodey hypothesizes that the ineffectiveness of vaccines may arise from loss of target antigens on tumor cells or microevolution of cells resistant to attack by the immune system.

Ezzel describes identification of various tumor specific antigens (i.e. MAGE-1, MART-1, MAGE 2, MAGE 3, GAGE and BAGE) and their use in cancer vaccine therapy. Although Ezzell suggests the potential insufficiency of a single peptide to trigger a immune

response strong enough to inhibit tumors, the reference suggests that there may be more effective ways to prompt cancer patients' immune systems to reject tumors (page 49, left column, first paragraph).

Spitler is characterized as describing the ineffectiveness of cancer vaccines. The Examiner cites the views given in Spitler of "practicing oncologists" and "venture capitalists" as supporting the nonenablement of the claimed compositions.

Treatments described in Bodey, Ezzel, and Spitler are drawn to vaccinations with tumor antigens. In contrast, instant claims are drawn to methods of inhibiting non-T cell tumor growth by using CTLA-4 blocking agent and a self antigen preparation. Consequently, the references are not germane for inferring the unpredictability or ineffectiveness of the claimed method in inhibiting tumor growth. Nonetheless, the Examiner equates the claimed methods to those described in Bodey, Ezzell, and Spitler.

The Applicants respectfully remind the Examiner that the initial burden for establishing nonenablement rests on the Patent Office. In order to make a rejection, the Examiner must establish a reasonable basis to question the issue of enablement. See M.P.E.P. § 2164.04; see also In re Wright, 999 F.2d 1557, 1563, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). In this process, the "specification which contains the teachings of the manner and process of making and using the claimed subject matter must be taken as being in compliance with the enablement requirement unless there is a reason to doubt the objective truth of the statements contained therein . . . ." See M.P.E.P. § 2164.04.

In the instant case, the Examiner does not advance a sufficiently reasonable basis to doubt the teachings of the specification or question the objective truth of the statement regarding the effectiveness of the claimed method in inhibiting tumor growth. The references of Bodey, Ezzell and Spitler are not material to the claimed subject matter since no CTLA-4 blocking agents nor immune response stimulating agents comprising self antigens are discussed or used in the therapies described. In this regard, extrapolating the results of Bodey, Ezzell and Spitler to the claimed method is both unreasonable and

inappropriate. Accordingly, an unreasonable inference has been drawn about the unpredictability of the effectiveness of the claimed methods.

After concluding the general unpredictable operability of cancer vaccines, the Examiner determines that unpredictability specifically arises from establishment of immune tolerance or immune suppression as described by Boon, T. et al. (1992) Advances in Cancer Res. 58: 177-210 (hereinafter "Boon"), Timmerman, J.M. et al. (1999) Ann. Rev. Med. 50: 507-29 (hereinafter "Timmerman"), and Areci, R,J. (1998) J. Mol. Med. 76: 80-93 (hereinafter "Areci"). Boon is cited for the proposition that the large tumor burden of cancer patients often leads to immune tolerance, which may defeat therapies based on immunization. Timmerman is cited for the proposition that impaired antigen presenting functions of dendritic cells (DCs) or presentation of antigens in a toleragenic manner caused by IL-10 (secreted by some tumors) renders the claimed composition potentially ineffective. Areci is cited for the proposition that absence of tumor specific antigens or costimulatory molecules (i.e. ligands of CD28 receptor such as B7) on some tumor cells creates unpredictability in inhibiting tumors by immunotherapy.

Boon suggest the presence of tumor specific antigens in most tumors and describes their use in priming the immune response to mediate tumor rejection. Rejection of tumors is indeed observed for some types of rodent tumors. Boon indicate the possibility of large tumor burdens tolerizing or at least depressing the capability of T-cells to respond against tumors and suggest that effective rejection of tumors by immunization in some patients may require eliminating tolerance or non-responsiveness (page 204, second paragraph).

Timmerman describe the role of dendritic cells (DC) in presenting tumor antigens to activate naive T-cells and summarizes the results of immunotherapy based on DC cells primed with tumor antigens. Although Timmerman suggest possible impairment of DC mediated antigen presentation, for example in tumors secreting IL-10, the rejection of tumors in several clinical trials (Non-Hodgkins Lymphoma and malignant melanoma) leads Timmerman to state

"even the earliest attempts at DC vaccination for cancer immunotherapy have demonstrated efficacy against several human cancers." (page 524, paragraph 3).

Areci describe treating acute myelogenous leukemia with anti-tumor vaccines by expressing costimulatory ligand B7 in the tumor cells to overcome potential deficiencies in proper antigen presentation or deficient levels of costimulatory molecules on tumor cells. Areci teach that expressing the B7 costimulatory ligand in tumor cells leads to tumor rejection.

Applicants submit that Boon, Timmerman or Areci do not support the assertions of unpredictability or ineffectiveness of the claimed method. The methods used for immunotherapies in Boon, Timmerman, and Areci comprised tumor antigen or expression of B7 costimulatory molecule. Neither Boon, Timmerman, or Areci examined CTLA-4 blocking agents and immune response stimulating agent comprising self antigen as part of the immunotherapy regimen. Thus, the Examiner has improperly equated the results of methods different from the claimed methods to demonstrate an alleged unpredictability arising from immune tolerance or suppression.

In this regard, the above-referenced comment in Boon is germane since the claimed combination of a CTLA-4 blocking agent and a self antigen preparation serves to break immune tolerance against a self antigen and can activate an autoreactive immune response against tumor cells expressing the self antigens, as demonstrated by the examples in the instant specification. The Examiner has failed to consider these teachings and appears to dismiss the exemplified inhibition of tumor growth by the claimed methods without providing a sufficient reason to doubt the objective truth of the results. Thus, in concluding the ineffectiveness and unpredictability of the claimed compositions, the Examiner has not fully considered the evidence of record, and instead relies on references which do not materially address the credibility of the claimed subject matter. Sufficient factual evidence has not been advanced to rebut the statements of the disclosure or to rebut the results of tumor growth inhibition described in the specification.

In short, the Examiner has collected a series of largely inapposite references which fail to address or call into question the efficacy of the claimed method, i.e. the combination of CTLA-4 blocking agents with self antigen preparations to break immune tolerance. In fact, contrary to the Examiner's position, several of the cited references clearly indicate that cancer immunotherapy in general, and tumor vaccination in particular, can be effective in inhibiting tumor growth. Accordingly, the Examiner has failed to properly establish the alleged unpredictability of the claimed method in inhibiting tumor growth.

- *Ineffectiveness of Cancer Vaccines and Enablement of Claimed Compositions*

Upon concluding the unpredictability of cancer vaccines, the Examiner arrives at a determination that the claimed compositions are not enabled because cancer vaccines are ineffective in view of the results of Lee et al. (1999) J. Immunol. 163: 6292-6300 (hereinafter "Lee"), Zaks, et al. (1998) Cancer Res., 58: 4902-4908 (hereinafter "Zaks"), Gao, et al. (2000) J. Immunotheraphy 23: 643-653 (hereinafter Gao), and Timmerman et al. (supra). Applicants respectfully traverse.

Lee teach that treatment of melanoma patients with melanoma antigen (MA) peptide leads to moderate expansion of MA-specific T-cell precursors (TCPF), which show increased susceptibility to *in vitro* stimulation with the peptide. This expansion of peptide specific T-cells, however, is not always correlated with evident clinical regression of the melanoma. (page 6299, right column, third paragraph).

Similarly, Zak teach treating cancer patients with HER-2/neu peptide, which is the immunodominant epitope on the HER-2/neu antigen found in a variety of tumors. Following treatment of the patients with peptide, peripheral blood mononuclear cells (PBMC) are isolated from the patients and stimulated *in vitro* with peptide to expand peptide specific cytotoxic T-cells. These activated T-cells, however, fail to act against HER-2/neu$^{+}$ tumor cells *in vitro* suggesting activation of T-cells with tumor antigen does not necessarily lead to effective immune mediated killing of the cancer cells.

Gao teach administering mitomycin inactivated tumor cells to mice harboring tumors to enhance immune mediated killing of non-IL-2 sensitive tumor cells. Although vaccination with inactivated tumor cells elicits IL-2 responsiveness in non IL-2 inhibited tumor cells, there was no significant inhibition of tumor growth by the vaccination.

Timmerman has been described above. The Office Action alludes to the results of Mukherji, B. et al., (1995) Proc. Natl. Acad. Sci. USA. 92: 8078-82 (hereinafter "Mukherji") and Wen, Y.J. et al. (1998) Clin. Cancer Res. 4: 957-62 (hereinafter "Wen"), both of which are cited in Timmerman, to support the alleged ineffectiveness of the claimed composition. Mukherji disclose that injection of GM-CSF and peptide treated monocytes elicits melanoma reactive cytotoxic T-cells in melanoma patients but fails to induce a therapeutic response. Wen disclose that a single patient vaccinated with idiotype-pulsed monocyte-derived DC displayed T-cell proliferation, but failed to provide significant clinical response.

Similar to the other references cited in the Office Action, Lee, Zak, Gao, Mukherjee, and Wen teach variations on vaccination with tumor antigen. These references do not describe or address methods using CTLA-4 blocking agent and a self antigen preparation to break immune tolerance, and are therefore largely inapposite.

Notwithstanding the inapplicability of the references to the claimed methods, Timmerman contradicts the conclusion of ineffectiveness of cancer vaccines since positive outcomes are seen for Non-Hodgkin's Lymphoma (page 517-18) and malignant melanoma (page 519-520). Based on the early trials, Timmerman states "it is encouraging that even the earliest attempts at DC vaccination for cancer immunotherapy have demonstrated efficacy against several human cancers." (page 524, third paragraph). Thus, the conclusion of Timmerman suggests the efficacy of cancer immunotherapy rather than inefficacy.

Nevertheless, the Examiner further holds the claimed methods nonenabled because the effectiveness of the methods allegedly rely on homogeneous expression of the "self-antigen" while "many tumors are a heterogeneous mosaic of cancer cells that either do or do not express a particular self-antigen" The Examiner concludes "[t]his fact, alone, severely limits

the potential for the successful use of most tumor vaccines, particularly those that are composed of a single tumor-associated antigen." Applicants respectfully disagree.

The Examiner appears to suggest that the method relies on expression of a single self antigen on the tumor cell. Cells, including normal cells and tumor cells, express a variety of self antigens capable of use in the subject method. Moreover, the Examiner does not point to references to support the alleged ineffectiveness of these self antigens when combined with a CTLA-4 blocking agent. Nor is there sufficient evidence to doubt the exemplary inhibition of tumor growth as described in the specification.

Under the M.P.E.P. § 2164.05, a determination of enablement should always be based on the weight of all the evidence. In this case, the Examiner has taken only those parts of the art allegedly supporting nonenablement and dismissed both clearly supportive data provided in the specification.

In summary, the Applicants submit that the Examiner has not provided a reasonable basis to demonstrate the alleged ineffectiveness of the claimed method to render it nonenabled. The references cited to support nonenablement are not material for determining the efficacy of the claimed subject matter because none of the references describe the use of CTLA-4 blocking agents in combination with self antigen preparations to stimulate an autoreactive T cell response against tumor cells expressing self antigen. Thus, the art and the reasoning advanced to support the Examiner's position fails to produce any reason to doubt the objective truth of the statements in the disclosure and the exemplary inhibition of tumor growth exhibited by the claimed method. Accordingly, Applicants submit that the claimed methods are enabled under § 112, first paragraph.

*Additional Reasons Regarding Enablement of Claimed Compositions*

The Examiner advances additional reasons why the claimed methods are nonenabled. Sotomayor, E.M. et al. (1999) Proc. Natl. Acad. Sci. USA 96: 11476-11481 (hereinafter "Sotomayor") is offered to show that sufficient reasons exist to doubt the credibility of the

claimed compositions since in Sotomayor CTLA-4 blocking agent did not prevent induction of tolerance to tumor cells. In a related issue, the Examiner contends a person skilled in the art would "not be able to use the invention with any degree of success" in view of Yang et al. (1997) Cancer Res. 57: 4036-4041 (hereinafter "Yang") because anti-tumor effect of CTLA-4 blockade is observed when administered into early tumor-bearing mice but is not effective when administered into late tumor-bearing mice. Applicants respectfully disagree.

Sotomayor disclose that early administration of CTLA-4 blocking antibody and tumor antigen to early tumor-bearing mice results in enhanced priming of T-cells responsive to *in vitro* antigen stimulation, which correlate with rejection of established tumors in early-tumor bearing mice by combined CTLA-4 blockade and antigen stimulation. However, administration of CTLA-4 blocking agent and tumor antigen to late-tumor bearing mice result in T-cells relatively unresponsive to antigen stimulation, suggesting induction of tolerance to antigen. Consequently, Sotomayor suggest that populations of T-cells with low affinity for antigen would be the cells most responsive to combined CTLA-4 blockade and antigen stimulation and thus constitute a basis for mediating tumor rejection (page 11481, right column).

Yang disclose effects of administering CTLA-4 blocking agent but without tumor antigen. Treatment of early tumor bearing mice resulted in enhanced induction of antitumor T-cell response and regression of established tumors. However, in Yang administration in late tumor-bearing mice did not enhance T-cell responses against established tumors.

In contrast to the asserted ineffectiveness of CTLA-4 blockade for any tumor, Yang disclose that CTLA-4 blocking agent is effective in inhibiting tumor growth, especially when given to early tumor-bearing mice. Although Yang suggests lack of enhancement of response in late tumor bearing mice in the particular tumor model under consideration, Yang is not dispositive of the effectiveness of the claimed methods since Yang administers CTLA-4 blocking agent without a self antigen preparation as presently-claimed.

Sotomayor also contradicts the Examiner's assertion of ineffectiveness by disclosing the ability of combined CTLA-4 blockade and antigen stimulation to stimulate rejection of established tumors in early tumor bearing mice. Rather than demonstrating ineffectiveness of the claimed methods, Sotomayor state the importance of CTLA-4 blockade for T-cell populations with low affinity for antigen:

> its response to immunization may be modest when conventional vaccine strategies are used, further underscoring the utility of CTLA-4 blockade in its ability to lower the threshold required for T-cell priming.

(page 11476, right column, third paragraph). Thus, Satomayor suggest effectuating rejection of tumor cells by combined CTLA-4 blockade and antigen stimulation to enhance responsiveness of T-cells that are not rendered tolerant to tumors due to low affinity for tumor antigen (page 11476, right column, third paragraph). In concert with Sotomayor, the claimed methods use CTLA-4 blocking agent and immune stimulating agent comprising a self antigen to activate low avidity T-cells and naive T cells against self antigen, thus abrogating the natural tolerance existing against self antigens. Therefore, rather than creating doubts about the effectiveness of the claimed methods, Sotomayor describe a basis for the effectiveness of the presently-claimed method.

The Applicants respectfully remind the Examiner that in determining enablement, absolute certainty is not required by law: "[t]he evidence provided by the applicant need not be conclusive but merely convincing to one skilled in the art." M.P.E.P. § 2164.05. Moreover, Federal Circuit states

> the presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. The standard is whether a skilled person could determine which embodiments that were conceived but not yet made, would be inoperative or operative with expenditure of no more effort that is normally required in the art.

See Atlas Powder Co. v. E.I. du Pont de Nemours & Co., 750 F.2d at 1577 (Fed. Cir. 1984). In the instant case, the Examiner appears to require complete effectiveness of the claimed method for all tumors under all conditions, *i.e.* a complete cure for cancer. This standard,

however, is not the proper standard under the law. The patent claims are directed to inhibition of tumor growth, not eradication. Moreover, the Examiner advances unsupported opinion that effectiveness of the claimed method requires diagnosis of tumors in patients "immediately following the onset of tumorigenesis, which almost never occurs" when immune system is not rendered tolerant to tumor antigen. However, the claimed methods are drawn to breaking the threshold tolerance of T-cells against self antigens. Moreover, as the Examiner's opinion is not supported by factual evidence or scientific data, it does not rebut the *in vivo* data of tumor growth inhibition provided in the disclosure.

The Examiner also argues that the claimed methods are nonenabled in view of Christadoss et al (Clinical Immuno. 94: 75087) (hereinafter "Christadoss"), Sullivan et al. (1998) FASEB J. 12: A1092 (hereinafter "Sullivan"), Zhu et al. (2001) J. Immunol. 115: 111-17 (hereinafter "Zhu") and Chambers et al. (1997) Proc. Natl. Acad. Sci. USA 94: 9296-301 (hereinafter "Chambers"), arguing that CTLA-4 blockade would potentially cause greater harm to the patient than benefit from treatment.

Under the patent laws, "while an applicant may on occasion need to provide evidence to show that an invention will work as claimed, it is improper for Office personnel to request evidence of safety in the treatment of humans." M.P.E.P § 2107.03. Moreover, "the Office must confine its review of patent applications to the statutory requirements of the patent law. Id. In accordance, the Federal Circuit has held

> [t]esting for the full safety and effectiveness . . . is more properly left to the Food and Drug Administration (FDA). Title 35 does not demand that such human testing occur within the confines of Patent and Trademark Office (PTO) proceedings.

See Scott v. Finney, 34 F.3d 1058, 1063, 32 USPQ2d 1115, 1120 (Fed. Cir. 1994). In view of this clear standard set by the Federal Circuit, the Examiner's proposed requirement of enablement is not in accordance with the law. Enablement of the methods does not require a showing of the safety of the claimed subject matter when used to inhibit tumor growth in mammals. The Examiner's rejection on this basis is clearly improper.

Nevertheless, the references of Christadoss, Sullivan, and Zhu support the basis of the claimed method since administering CTLA-4 blocking agent and immunogens similar to "self antigens" can elicit experimental autoimmune reactions. Thus, by directing T-cells against self antigens expressed on tumor cells, the claimed methods allow inhibition of tumor growth otherwise ineffectively inhibited through immune response against tumor antigens.

The Examiner finds the claimed methods further nonenabled in view of Gribben et al. (1994) Blood, 84: 397a (hereinafter "Gribben"), which suggest induction of T-cell apoptosis upon engagement of CTLA-4 receptors. Gribben is allegedly supported by the observations of Anderson, D. et al. (2000) Nature Med. 6: 211-214 (hereinafter "Anderson"). Applicants respectfully disagree.

The Examiner appears to suggest the "possibility" that CTLA-4 mediated apoptosis of "self-antigen reactive T-cells" induced by CTLA-4 blocking agent renders the claimed methods ineffective in inhibiting tumor growth. Gribben describe *in vitro* experiments showing crosslinking of CTLA-4 receptor with anti-CTLA-4 antibodies induces apoptosis in previously activated T-cells. The Examiner, however, does not provide factual evidence that these observations manifests in the ineffectiveness of the claimed methods *in vivo* in a mammal. Gribben in another publication also show induction of cell death is more complicated since presence of interleukin 2 (IL-2) abrogates apototic effect of CTLA-4 receptor crosslinking (Exhibit H, Gribben, J.G. et al. (1995) Proc. Natl. Acad. Sci. USA. 92: 811-15).

The Examiner suggests Anderson supports Gribben in showing CTLA-4 blockade results in T-cell death. Anderson discloses that CTLA-4 blocking agent enhances or inhibits different T-cells depending on the level of costimulation with antigen. Anderson, however, makes no suggestion that lack of expansion arises from programmed cell death. Moreover, in direct contrast to the opinions of the Examiner, Anderson demonstrate CTLA-4 blockade and stimulation by weak antigen leads to expansion of responsive T-cells (page 213, right column, fourth paragraph), which is the desired effect in the claimed subject matter.

More important to the issue of enablement, there is no showing that the *in vitro* observations of Gribben creates any reason to doubt the objective truth or accuracy of the actual data provided in the disclosure demonstrating the inhibitory effect of the claimed methods on tumor growth *in vivo*. The Examiner fails to provide a reasoned analysis of why the inhibition of tumor growth by CTLA-4 blocking agent as exemplified in Example 2, Example 6, Example 7, Example 8 and Example 9 does not reflect the effect of CTLA-4 blocking agent. Further, there is no explanation of why the autoreactive T cell response against both normal and tumor cells in Example 10 at pages 47-58 and in Example 11 at pages 64-65 does not reflect the efficacy of the claimed method. Accordingly, Applicants submit the uncertainties advanced by the Examiner are not supported by the evidence of record and are wanting in sufficiency to meet the burden of nonenablement for the claimed subject matter.

*Enablement of Inhibiting Tumor Growth in Mammals and Cancer Therapy*

Finally, since the claimed methods are allegedly nonenabled for inhibiting tumors of any kind, the Examiner finds claim methods nonenabled for treating any tumor growth in any mammal, including treating human patients. Furthermore, the Examiner contends the claims are nonenabled for human patients because the specification teaches use of the claimed method on a mouse but fails to provide sufficient guidance for use in human patients in a clinical setting. Applicants respectfully traverse.

The Applicants have argued above that the Examiner has not advanced sufficient reasons to render claims 21, 23-28 and 31 nonenabling. The Examiner has applied references not material to the claimed methods and failed to provide adequate evidentiary support to reasonably question the objective truth of the statements in the disclosure regarding the effect of the claimed methods in inhibiting tumor growth. Thus, a *prima facie case* of nonenablement has not be been established. Moreover, the M.P.E.P states

> If a statement of utility in the specification contains within it a connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated, 35 U.S.C. is satisfied. . . . It is not necessary to

> specify the dosage or method of use if it is known to one skilled in the art that such information could be obtained without undue experimentation. If one skilled in the art based on knowledge of the compounds having similar physiological or biological activity would be able to discern an appropriate dosage or method of use without undue experimentation, this would be sufficient to satisfy 35 U.S.C. 112, first paragraph. The applicant need not demonstrate that the invention is completely safe.

M.P.E.P. § 2164.01(c). In addition, regarding the value of animal tests, the M.P.E.P instructs

> if one skilled in the are would accept the animal tests as being reasonably predictive of utility in humans, evidence from those tests should be considered sufficient to support the credibility of the asserted utility.

In view of the established standards, Applicants submit that the claimed methods are enabled for use in mammals, including human subjects. The considerable history of experience with cancer vaccines reflects a high degree of skill in the art for formulating and administering pharmaceutically suitable forms of immunotherapeutic agents. Consequently, given the guidance and direction provided in the specification, a person skilled in the art can determine proper methods of administration in human subjects and determine a dosage sufficient to inhibit tumor growth. In support of the high level of skill in the art, the Applicants direct the Examiner to reference of Timmerman, which describes a variety of clinical trials involving cancer vaccine therapy (i.e. page 522). In addition, Bodey describes various cancer vaccine treatments and clinical trials with human patients, for example clinical trials with anti-idiotype antibody therapy (page 2669, left column) and cytokine gene-modified tumor cells (page 2670, right column and page 2671).

Moreover, the disclosure provides considerable guidance and direction on use of the claimed methods in cancer immunotherapy. The specification describes various modes of administration, which include intravascular, subcutaneous, or peritoneal delivery (page 23, lines 21-30). Example 2 describes an exemplary mode of administration (i.e. intraperitoneal) effective in inhibiting tumor growth (page 35). The specification further provides a range of CTLA-4 dosage (i.e. 0.1 to 100 mg/kg of body weight), the related units of activity, the regimen of injections, and pharmaceutical media suitable for determining a proper, effective

dosage (page 24). The specification then discloses specific dosages for CTLA-4 blocking agent and immune response stimulating antigens which provide therapeutic effects (see specification under Example 2, Example 6, Example 7, Example 9, Example 10, and Example 11). Given the level of skill in the art, the *in vivo* data disclosed in the specification could be correlated with an effective dosage to administer to human subjects.

Thus, the guidance and direction given in the specification combined with the knowledge of those skilled in the art in regards to standard modes of administration, treatment regimens, and acceptable pharmaceutical mediums, renders the methods enabled for use in mammals and human patients. Accordingly, Applicants submit that the methods of claims 21, 23-28 and 31 are fully enabled for use in inhibiting tumors in humans and other mammals.

*Conclusions*

In view of the foregoing, Applicants submit that the Examiner has not established a *prima facie* case of lack of enablement. The specification provides sufficient direction and guidance in making and using the claimed CTLA-4 blocking agents and self antigen preparations. The effectiveness of the method in inhibiting tumor is not unpredictable nor does determining effectiveness for a particular tumor require undue experimentation. Moreover, no evidence has been advanced by the Examiner to doubt the objective truth of the effect of the claimed methods in inhibiting tumor growth in mammals. Thus, the Examiner's rejection for lack of enablement is clearly improper in this case. Accordingly, Applicants respectfully request withdrawal of the rejection under 35 U.S.C. § 112, first paragraph.

**Rejections Under 35 U.S.C. §112, second paragraph: written description**

Claims 21-25, 27-29 and 31 stand rejected under 35 USC § 112, first paragraph for inadequate written description. Specifically, the Examiner contends the specification fails to specify the identity of even one CTLA-4 blocking agent other than monoclonal 9H10 suitable

for use in the claimed methods. Thus, the Examiner concludes that Applicants were not in possession of the claimed genus of CTLA-4 blocking agents. Applicants respectfully disagree.

The Examiner correctly states that the written description requirement is separate and distinct from the enablement requirement. See Vas-Cath, Inc. v. Marhurkar, 935 F2d 1555, 1563-64, 19 USPQ2d 1111, 1115 (Fed. Cir. 1991). The public policy rationale for the written description requirement is to "disclose in the patent sufficient information to place in the public what the inventors claim is their invention." Id. at 1114. Accordingly, the specification is required to convey with reasonable clarity that the inventor had possession of the invention at the time of filing the application. Thus, compliance with the written description requirement is met either by an express or inherent disclosure and may be satisfied if the "specification contains a statement of appellants invention which is as broad as appellant's broadest claims . . . .". See In re Robins, 420 F.2d 452, 166 USPQ 552, 555 (CCPA 1970); see also In re Lukach, 442 F.2d 967, 969, 169 USPQ 795 (CCPA 1971); see also M.P.E.P. § 2163.

The Applicants submit that the specification provides sufficient description of CTLA-4 blocking agents to satisfy the written description standard. The specification describes CTLA-4 blocking agent as an agent characterized as specifically binding to the extracellular domain of the CTLA-4 receptor (specification page 8, lines 7-9). The sequence of the extracellular domain was known in the art at the time of the present invention and was readily available to the skilled artisan (see page 5, line 26-31; page 6, line 1-4). The blocking agents bind with an affinity of at least about 100 μM and blocks the binding of CTLA-4 to counter receptors, including CD80 and CD86 (page 8, lines 7-11). Moreover, the blocking agents, which are either monovalent or bivalent in structure, do not activate CTLA-4 mediated signaling (page 9, lines 5-7). The specification further recites that such blocking agents may comprise antibodies, peptides, small organic molecules, and peptidomimetics (page 10-14). Preferred blocking agents are antibodies (page 8, lines 6-17). Antibodies against human CTLA-4 or humanized antibodies are described on page 13, lines 13-14 and Example 4, page 40-43.

Moreover, the Applicants describe an exemplary blocking agent in 9H10 monoclonal antibody, which satisfies all the enumerated criteria of the claimed genus of CTLA-4 blocking agents.

The Examiner appears to suggest that satisfying written description under § 112, first paragraph requires recitation of the epitope specificity of the blocking agent or the amount of blocking agent that would provide efficacy. Nothwithstanding the statement in the disclosure specifically referring to blocking agents as binding the extracellular domain of CTLA-4, Applicants submit the requirement imposed is clearly improper. As stated by the Federal Circuit, the test is whether the Applicants have clearly conveyed with reasonable clarity that the inventor had possession of the invention at the time of filing the application. Since the specification provides specific descriptions of CTLA-4 blocking agents and describes an exemplary CTLA-4 blocking agent in 9H10, the Applicants have sufficiently conveyed with reasonable clarity the genus of CTLA-4 blocking agents, thus satisfying the written description requirement under § 112, first paragraph. Accordingly, Applicants respectfully request withdrawal of the rejection.

**Rejections Under 35 U.S.C. §112, second paragraph**

Claims 21-25, 27-29 and 31 stand rejected under 35 U.S.C. § 112, second paragraph as being indefinite. Specifically, the Examiner considers the claims lacking in a positive process step. Applicants respectfully traverse.

Claim 21 has been amended to recite a positive process step whereby, contacting at least one T cell with a self antigen preparation and CTLA-4 blocking agent is effective to break immune tolerance and stimulate an autoreactive T cell response against self antigen expressed on non-T cell tumors and normal cells. Accordingly, Applicants respectfully request withdrawal of the rejection.

Claims 21-25, 27-29, and 31 stand rejected under § 112, second paragraph because the Examiner contends that claim 21 is indefinite in reciting whether (1) the CTLA-4 blocking

agent specifically bind the extracellular domain of CTLA-4 or whether the CTLA-4 blocking agent is defined as having the characteristic of being able to bind specifically the extracellular domain of CTLA-4, (2) whether the claims require that CTLA-4 blocking agent specifically inhibit CTLA-4 signaling or whether CTLA-4 merely have the characteristic of being able to inhibit CTLA-4 signaling, and (3) whether the CTLA-4 blocking agent inhibits CTLA-4 signaling by binding the extracellular domain of CTLA-4 or if CTLA-4 is capable of both activities. Applicants respectfully traverse, since the proper grammatical construction of the referenced phrase as well as the common and ordinary definition of the term "characterize" provide a clear and definite meaning for the claimed subject matter.

Specifically, Claim 21 recites that the CTLA-4 blocking agent is "characterized as specifically binding to the extracellular domain of CTLA-4 and inhibitory of CTLA-4 signaling." The term "characterize" is defined as "to be a characteristic of." (Oxford English Dictionary, 2nd Ed. Vol. III, p. 33, Clarendon Press, Oxford, 1989). Thus, the common and ordinary definition the term "characterized" together with Applicants use of the conjunctive "and" plainly dictates that the claimed CTLA-4 blocking agent must have the characteristics of 1) binding to the extracellular domain and 2) inhibiting of CTLA-4 signaling, which is fully consistent with only two of the alternative definitions proffered by the Examiner and necessarily excludes the others.

An alternative way of phrasing this is to say that the CTLA-4 blocking agent must be "capable of" binding to the extracellular domain of CTLA-4 and inhibiting CTLA-4 signaling. The "characterized as" and "capable of" alternatives are synonymous, since a blocking agent that is capable of binding to the extracellular domain and inhibiting CTLA-4 signaling has those two characteristics, and vice versa. In any event, as indicated above Applicants respectfully submit that the common and ordinary definition of "characterized" together with the grammatical structure of the disputed portion of the claim clearly indicates what is being claimed. Accordingly, the Applicants respectfully request withdrawal of the rejection.

Claim 24 stands rejected under § 112, second paragraph because the Examiner considers use of the term "cytokine transduced" and "GM-CSF-transduced" indefinite. Applicants respectfully traverse.

Claim 24 has been amended to recite that the tumor cell is transduced with a nucleic acid construct capable of expressing a cytokine. Applicants submit that the claims is sufficiently definite under 35 U.S.C. 112, second paragraph. Accordingly, Applicants respectfully request withdrawal of the rejection.

**Rejections Under 35 U.S.C. § 102**

Claims 21-23 and 27 stand rejected under 35 U.S.C. § 102(a) as being anticipated by Leach, D.R. et al (1996) Science 271: 1734-1736 (hereinafter "Leach"). Applicants respectfully traverse.

Leach teach inhibiting tumor cell growth by administering CTLA-4 blocking agent concurrently or subsequent to inoculation of mice with murine carcinoma or fibrosarcoma cells. The CTLA-4 blockade and tumor cell inoculation leads to inhibition of tumor cell growth in the mice.

In contrast, claim 21 recites a method of inhibiting the growth of non-T cell tumors using a self antigen preparation and a CTLA-4 blocking agent, which is effective in breaking immune tolerance and simulating an autoreactive immune response against the self antigen on tumor cells and normal cells. Claim 26 enumerates certain purified self antigens suitable as immune response stimulating agents. Purified antigen is described in page 19, page 21-30, in part, as "protein isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host."

Anticipation of a claim requires that a single reference expressly or inherently disclose each and every element of the claim. See In re Paulsen, 31 USPQ2d 1671, 1673 (Fed. Cir. 1994); M.P.E.P. § 2131 (citing Richandson v. Suzuki Motor Co., 9 USPQ2d 1913, 1920 (Fed. Cir. 1989). In the instant case, Leach fail to teach use of self-antigen preparations in

combination with CTLA-4 blocking agents to break immune tolerance against self antigens. Rather, Leach teach administration of CTLA-4 blocking agent and tumor cells to permit activation of T cells directed against tumor cells expressing tumor antigen. Since Leach do not teach each and every element of claim 21, Leach cannot anticipate claim 21. As claims 23-28 and 31 ultimately depend from claim 21, Leach do not anticipate these dependent claims. In view of the foregoing, Applicants respectfully request withdrawal of the rejection of claims 21, 23, and 27 under 35 U.S.C. § 102(a).

Claims 21-25 and 27-29 stand rejected under 35 U.S.C. § 102(e) as being anticipated by Allison et al., U.S. Pat. No. 5,811,097A (hereinafter '097A"); while Claims 21-25, 27 and 28 stand rejected under 35 U.S.C. § 102(e) as being anticipated by Allison et al., U.S. Pat No. 5,855,887 (hereinafter '887). Applicant respectfully traverse.

Both '097A and '887 teach enhancing T-cell activation by the use of CTLA-4 blocking agents to block CTLA-4 inhibitory actions on T-cell activation. Patents '097A and '887 further teach the combination of CTLA-4 blocking agents with 1) cytokines or other proteins known to enhance T cell proliferation, 2) the transfection of tumor cells or tumor-infiltrating lymphocytes with genes encoding for various cytokines or cell surface receptors, 3) tumor specific host T cells, and 4) tumor antigens including, *inter alia*, purified protein or lysates from tumor cells. ('097A, col. 8, line 37 through col. 9, line 50; '887, col. 8, line 25 through col. 9, line 35). In one aspect, this method inhibits growth of non-T cell tumors by enhancing immune activity against tumor antigens expressed on tumor cells.

Claim 21, as described above, is drawn to the use of self antigen preparations as an immune response stimulating agent. The claim recites that the self antigen preparation in combination with a CTLA-4 blocking agent will break immune tolerance to the self antigen and direct the immune response against self antigens expressed on both tumor cells and normal cells. The instant specification exemplifies a combined tumor and autoimmune response against melanocytes (vitiligo, Example 10 at pages 56-57) and prostate cells (prostatitis, Example 11 at pages 59 & 64-66). The specification further discloses the

combination of a purified murine self antigen (gp100) and a CTLA-4 blocking agent to specifically break immune tolerance against cells expressing this particular self antigen, thereby inhibiting growth of tumor cells expressing gp100 in mice.

'097A and '887 do not teach or suggest an autoreactive T cell response against self antigens present on both normal and tumor cells as presently claimed. Since '097A and '887 fail to teach each and every element of claim 21, they cannot properly anticipate claim 21. As claims 23-28 and 31 ultimately depend from claim 21, they also do not anticipate these dependent claims. Accordingly, Applicants respectfully request withdrawal of the rejection under § 102(e) over US Pat. No. 5,811,097A and US Pat. No. 5,855,887.

**Rejections Under 35 U.S.C. § 103(a)**

Claims 21-25, 27-29 and 31 stand rejected under 35 U.S.C. § 103(a) as being rendered obvious over Leach et al (1996) Science, 271: 1734-36, in view of Heslop (1994) Bailleires Clinical Haemotology 7: 296-306 (hereinafter "Heslop"), Sussman, J.J. (1994) Ann. Surg. Oncol. 1: 296-306 (hereinafter "Sussman"), and Wallack, et al (1992) Mt. Sinai. J. Med. 59: 227-33 (hereinafter "Wallack"). Applicants respectfully traverse.

The teachings of Leach have been described above.

Heslop teach cytokine gene transfer into tumor cells or immune system effector cells and subsequent transfer of these cells into patients to increase levels of cytokine at tumor sites, thereby activating immune response against tumor cells

Sussman teach adoptive immunotherapy in which immune system cells (i.e. lymphocytes) obtained from patients are activated *ex vivo*, for example with interleukin-2, and introduced back into the patient to inhibit tumor cell growth.

Wallack teach use of viruses to infect tumor cells to form viral lysates of tumor cells. These viral oncolysates are administered to the patient to enhance immune response against tumor cells.

In rejecting claims for obviousness under 35 U.S.C. § 103(a), the Examiner bears the burden of establishing a *prima facie* case of obviousness. See In re Bell, 26 USPQ2d 1529 (Fed. Cir. 1993); M.P.E.P § 2142. To establish a prima facie case of obviousness, three criteria must be met: (1) the prior art must provide one of ordinary skill with a suggestion or motivation to modify or combine the teachings of the references relied upon by the Examiner to arrive at the claimed invention; (2) the prior art must provide one of ordinary skill in the art with a reasonable expectation of success; and (3) the prior art, either alone or in combination, must teach or suggest each and every limitation of the claims. The teaching or suggestion to make the claimed invention, as well as the reasonable expectation of success, must come from the prior art, not Applicant's disclosure. See In re Vaeck, 20 USPQ2d 1438 (Fed. Cir. 1991); M.P.E.P. § 706.02(j). If any one these criteria are not met, *prima facie* obviousness is not established.

As indicated above, Leach do not teach nor suggest the use of self antigen preparations in combination with CTLA-4 blockade to abrogate immune tolerance against self antigens and stimulate an autoreactive T cell response. Leach is directed to enhancement of immune response against tumor antigens by administering CTLA-4 blocking agent and tumor cells. This deficiency in the disclosure of Leach is not overcome by its combination with Heslop and Wallack, which merely teach use of tumor cells or tumor cell lysates, nor by combination with Sussman, which merely teaches cytokine treatement as the immure response stimulating agent. Heslop, Wallack and Sussman also fail to teach or suggest administration of self antigen preparations in combination with CTLA-4 blockade to direct immune response against self antigens present on both normal and tumor cells. As the cited references fail to teach or suggest every limitation of claim 21, the references either alone or in combination, fail to render the claim 21 *prima facie* obvious. As claims 23-28 and 31 ultimately depend from claim 21, the references fail to render these claims obvious. Accordingly, Applicants respectfully request withdrawal of the rejections under 35 U.S.C. § 103(a).

Claims 21-25, 27-29 and 31 stand rejected under 35 U.S.C. § 103(a) as being rendered obvious over U.S. Pat No. 5,811,097A and/or U.S. Pat. No. 5,855,887. Applicants respectfully traverse.

Applicants have described the teachings of the cited patents above. The patents fail to teach or suggest the claimed combination of a self antigen preparation and CTLA-4 blockade to abrogate immune tolerance to self antigens present on both normal and tumor cells. Since '097A and '887 fail to teach or suggest every limitation of claim 21, they also fail to render the claim *prima facie* obvious. As claims 23-28 and 31 ultimately depend from claim 21, the '097A and '887 patents fail to render these claims obvious. Accordingly, Applicants respectfully request withdrawal of the rejections under 35 U.S.C. § 103(a).

**Obviousness Type- Double Patenting**

The Examiner contends the following claims rejected under judicially created obviousness-type double patenting: claims 21-25 and 27-29 and 31 stand rejected as being unpatentable over claims 1, 3, 4, 6, 8, 18 and 20 of U.S. Pat. No. 6,051,227A. Claims 21-23, 25, 27 and 29 stand rejected over U.S. Pat. No. 6,051,227 in view of Heslop (1994) Baillieres Clinical Haemotology 7: 135-51. Claims 21-25, 27, 29 and 31 stand rejected as being unpatentable over claims 1, 8, and 20 of U.S. Pat. No. 5,855,877A in view of Heslop (supra). Claims 21-23, 25, 27, 29 and 31 stand rejected as being unpatentable over claims 1 and 6-11 of U.S. Pat. No 5,811,097A. Claims 21-23, 25, 27, 29, and 31 stand rejected as being unpatentable over claims 1 and 6-11 of U.S. Pat. No. 5,811,097A in view of Heslop (supra). Applicants respectfully traverse.

Applicants respectfully submit that the claims as presently amended together with the foregoing arguments relating to the cited prior art references are sufficient to overcome the Examiner's obviousness-type double patenting rejections. As explained above, there is no disclosure in any of the cited references of a method of inhibiting the growth of non-T cell tumors by combining a self antigen preparation with a CTLA-4 blocking agent to break

immune tolerance to the self antigen and direct an autoreactive T cell response against the self antigen on both normal and tumor cells.

## CONCLUSION

The Applicants submit that all pending claims of the above referenced application are in compliance with all the requirements of patentability and are in condition for allowance. Accordingly, early notification of such allowance is earnestly solicited.

Attached hereto is a marked up version of the changes made to the claims by the "AMENDMENT AND REPLY TO OFFICE ACTION." The attached page is captioned "VERSION WITH MARKINGS TO SHOW CHANGES MADE." In addition, an Appendix of the pending claims is attached for the Examiner's convenience.

If after review, the Examiner feels there are further unresolved issues or determined that prosecution of the above reference application would benefit from a telephone interview, the Examiner is invited to call the undersigned attorney at (415) 781-1989.

Respectfully submitted,

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## VERSION WITH MARKINGS TO SHOW CHANGES

21. A method for inhibiting the growth of non-T cell tumor cells in a mammalian host, the method comprising:

contacting at least one T cell of said host with a [first immune response stimulating agent]self antigen preparation and a CTLA-4 blocking agent characterized as specifically binding to the extracellular domain of CTLA-4 and inhibitory of CTLA-4 signaling;

wherein said contacting is effective to [inhibit the growth of non-T cell tumor cells in said host] break immune tolerance and stimulate an autoreactive T cell response against a self antigen expressed on said non-T cell tumor cells and normal cells.

23. The method of Claim [22] 21, wherein said self antigen preparation comprises a tumor vaccine.

24. The method of Claim 23, wherein said tumor vaccine comprises [cytokine transduced] tumor cells transduced with a nucleic acid construct capable of expressing a cytokine.

25. The method of Claim [22] 21, wherein said self antigen preparation comprises tumor cell lysates.

26. The method of Claim 21, wherein said self antigen preparation comprises a puriifed tissue specific antigen selected from the group consisting of tyrosinase, trp1, trp2, melanA/MARTI, gp100, prostatic acid phosphatase (PAP), prostate specific membrane antigen (PMSA), prostate stem cell antigen (PSCA), prostase, and Her2/neu.

27 The method of Claim 21, wherein said contacting step comprises administering said [first immune response stimulating agent]self antigen preparation and said CTLA-4 blocking agent to said mammalian host simultaneously or sequentially.

31. The method of Claim 21, comprising contacting said mammalian T cell with [a second] an immune response stimulating agent either simultaneously or sequentially.

## APPENDIX OF PENDING CLAIMS

21. A method for inhibiting the growth of non-T cell tumor cells in a mammalian host, the method comprising:

contacting at least one T cell of said host with a [first immune response stimulating agent]self antigen preparation and a CTLA-4 blocking agent characterized as specifically binding to the extracellular domain of CTLA-4 and inhibitory of CTLA-4 signaling;

wherein said contacting is effective to [inhibit the growth of non-T cell tumor cells in said host] break immune tolerance and stimulate an autoreactive T cell response against said self antigen expressed on said non-T cell tumor cells and normal cells.

23. The method of Claim [22] 21, wherein said self antigen preparation comprises a tumor vaccine.

24. The method of Claim 23, wherein said tumor vaccine comprises [cytokine transduced] tumor cells transduced with a nucleic acid construct capable of expressing a cytokine.

25. The method of Claim [22] 21, wherein said self antigen preparation comprises tumor cell lysates.

26. The method of Claim 21, wherein said self antigen preparation comprises a puriifed tissue specific antigen selected from the group consisting of tyrosinase, trp1, trp2, melanA/MARTI, gp100, prostatic acid phosphatase (PAP), prostate specific membrane antigen (PMSA), prostate stem cell antigen (PSCA), prostase, and Her2/neu.

27 The method of Claim 21, wherein said contacting step comprises administering said [first immune response stimulating agent]self antigen preparation and said CTLA-4 blocking agent to said mammalian host simultaneously or sequentially.

28. The method of Claim 21, wherein said contacting step occurs *ex vivo* and said at least one T cell is administered to said host.

31. The method of Claim 21, comprising contacting said mammalian T cell with [a second] an immune response stimulating agent either simultaneously or sequentially.

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# Peptide ligands for a sugar-binding protein isolated from a random peptide library

(peptide diversity/fd bacteriophage/concanavalin A/affinity panning)

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ABSTRACT **Peptide ligands for the carbohydrate-binding protein concanavalin A (Con A) have been identified by screening a large, diverse peptide library expressed on the surface of filamentous phage. A dodecapeptide containing the consensus sequence Tyr-Pro-Tyr was found to bind Con A with an affinity (dissociation constant, $K_d$) of 46 $\mu$M, comparable to that of a known carbohydrate ligand, methyl $\alpha$-D-mannopyranoside ($K_d$ of 89 $\mu$M). In addition the peptide inhibited precipitation of the $\alpha$-glucan dextran 1355 by Con A. Given the complexity of oligosaccharide synthesis, the prospect of finding peptides that competitively inhibit carbohydrate-specific receptors may simplify the development of new therapeutic agents.**

Carbohydrate–protein interactions form the basis of a host of biological processes (1, 2). For example, the periplasmic monosaccharide-binding proteins of Gram-negative bacteria serve as receptors for transport and chemotaxis (3), while cell-surface lectins mediate such processes as cell–cell adhesion (4, 5) and lymphocyte migration through lymphoid tissues (6). Carbohydrate recognition is central to the enzymatic synthesis and degradation of polysaccharides, glycoproteins, and glycolipids that play essential roles in metabolism and in the maintenance of cellular structures. Carbohydrate–protein associations are also critical in certain cycles of viral infection. For example, binding of the hemagglutinin protein of human influenza virus to sialic acid residues on erythrocyte cell-surface glycoproteins represents the initial step in influenza infection (7).

Consequently, the development of potent inhibitors of carbohydrate-specific proteins may be of considerable importance in the generation of new therapeutic agents. However, the synthesis of complex carbohydrate ligands and analogs often requires many time-consuming, low-yielding steps. Chemical synthesis of oligosaccharides requires sophisticated strategies for protecting/deprotecting and assembling sugar monomers and for controlling product regiochemistry and stereochemistry (8–10). Enzymatic synthesis using glycosyltransferases has emerged as a useful alternative to chemical synthesis (11, 12). However, this approach is limited by the availability of enzymes with the appropriate specificities.

An alternative approach to the synthesis of polysaccharide ligands for carbohydrate-specific receptors is to ask whether peptides can be found that bind these proteins with high affinities. We describe a strategy for identifying novel peptide ligands for carbohydrate-binding proteins based on the screening of a large, highly diverse peptide library expressed on the surface of filamentous phage fd (13–16). The library consists of phage bearing random octapeptides fused to the amino terminus of the minor coat protein, pIII, and was screened by affinity purification on immobilized receptor. We chose as a model system the lectin Con A from jack bean, whose physicochemical properties have been extensively studied (17). Con A is a tetramer composed of four identical polypeptide chains consisting of 237 residues each. Con A, which interacts preferentially with oligosaccharides bearing terminal $\alpha$-linked mannose or glucose residues, is frequently employed in the purification and structural characterization of carbohydrates and glycoproteins and is also a lymphocyte mitogen. We report the isolation of peptide ligands for Con A that prevent binding of known monosaccharide ligands of the lectin and that inhibit Con A-dependent precipitation of polysaccharides. The prospect that specific ligands for carbohydrate-binding proteins can be assembled by coupling amino acid building blocks (rather than sugars) should greatly reduce the synthetic difficulties associated with inhibitor design.

## MATERIALS AND METHODS

**Reagents and Peptides.** Con A was obtained from Sigma or Calbiochem, biotinylated Con A from Boehringer Mannheim, and Con A-Sepharose from Pharmacia. Restriction enzymes, T4 DNA ligase, and T4 kinase were obtained from New England Biolabs or GIBCO/BRL. All other chemicals were obtained from Sigma. Oligonucleotides were synthesized with an Applied Biosystems PCR-mate and gel-purified. Peptides were synthesized on a MilliGen (Bedford, MA) synthesizer using fluorenylmethoxycarbonyl and *t*-butyl protecting groups and trifluoroacetic acid deprotection and were purified to >95% purity by reverse-phase HPLC using water/acetonitrile/0.1% trifluoroacetic acid as eluant. Composition of the purified peptides was confirmed by amino acid analysis and mass spectrometry. UV–visible spectra were recorded on a Cary 3 spectrophotometer. Equilibrium dialysis was performed with a Spectrum equilibrium dialysis chamber.

**Construction of a Random Octapeptide Library in fd Phage.** The random octamer library, constructed according to the protocol of Cwirla *et al.* (16), was kindly provided by W. J. Dower. Briefly, a collection of oligonucleotides encoding all possible octapeptides fused to the spacer peptide Ala-Ser-Gly-Ser-Ala (ASGSA) was synthesized as described (16) by using the nucleotide sequence 5′-C-TCT-CAC-TCC-NNK-NNK-NNK-NNK-NNK-NNK-NNK-NNK-GCA-AGT-GGC-TCT-GCT-ACT-GTT-GAA-AGT-TGT-3′ (oligonucleotide ON-141), where N is A, C, G, or T (equimolar) and K is G or T (40:60). Ten electrotransformations, each using 13.5 $\mu$g of ligated vector DNA and 200 $\mu$l of electrocompetent *Escherichia coli* MC1061 cells yielded, after 1 hr of nonse-

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lective outgrowth, a library of $1.4 \times 10^9$ transformants (75% producing infective phage). The cells were grown under tetracycline selection in 3 liters of L broth to amplify the library, and the phage were isolated as described (16).

**Affinity Purification.** Affinity purification was performed utilizing two different attachment strategies for the Con A. In the first, biotinylated Con A [20 μg/ml in Con A buffer (CAB: 50 mM NaCl/20 mM Mops, pH 6.8/2 mM $MgCl_2$/2 mM $CaCl_2$/0.2 mM EDTA)] was bound in 96-well microtiter plates coated with streptavidin (20 μg/ml in phosphate-buffered saline). Unbound Con A was removed by three 5-min washes with CAB. In the second strategy, commercially available Con A covalently attached to cyanogen bromide-activated Sepharose was used. Phage ($\approx 10^{10}$ infectious particles) were added to each well of the microtiter plate or to 20 μl of Con A-Sepharose in a microcentrifuge tube, in a final volume of 200 μl of CAB. Phage were allowed to bind for 1 hr at room temperature on a rocking platform. Unbound phage were removed by three 5-min washes with CAB (1 ml). Adherent phage were eluted with either 200 mM methyl α-D-mannopyranoside, 1% yeast mannan, or 100 mM citrate buffer (pH 3.0) for 30 min at room temperature. Eluted phage were amplified as described (16). This panning protocol was then repeated twice.

After three rounds of panning individual amplified phage clones were tested for their ability to specifically bind Con A as determined by ELISA. Biotinylated Con A was bound in the presence of bovine serum albumin (20 μg/ml in CAB) to microtiter plate wells as described above. Phage ($\approx 10^{10}$ transforming units) were added to each well and incubated for 1 hr. As a negative control, phage were added to identically treated wells from which Con A had been omitted. Unbound phage were removed by three washes with CAB. Bound phage were detected in a sandwich assay with rabbit anti-fd phage antiserum and a horseradish peroxidase-coupled goat anti-rabbit antibody (R. W. Barrett, S. E. Cwirla, M. S. Ackerman, A. M. Olson, E. A. Peters, and W. J. Dower, personal communication). Phage clones that displayed specific binding to Con A were amplified and their DNA was sequenced as described (16).

**UV Difference Spectroscopy.** Dissociation constants for peptide binding to Con A were determined at 20°C by the competition UV difference spectrophotometric method of Bessler *et al.* (18). Con A was 0.72 mg/ml in CAB and the competition difference spectra were recorded at 0.1 and 0.2 mM *p*-nitrophenyl α-D-mannopyranoside. Absorbance changes were measured 90 min after addition of peptide to ensure equilibration with the chromogenic sugar. The absorbance change ($\Delta A$) at 317 nm was plotted in the form $[(\Delta A_{max}/\Delta A) - 1][D]$ vs. [L] where $\Delta A_{max}$ is the absorbance change when the protein is saturated with *p*-nitrophenyl α-D-mannopyranoside, [L] is the concentration of the competing ligand, and [D] is the concentration of free chromogenic ligand. The competition dissociation constant $K_d$ is given by the product of the dissociation constant of the chromogenic sugar and the reciprocal slope of this linear representation of the data.

**Equilibrium Dialysis.** The peptide DVFYPYPYASGS was N-methylated with [$^{14}$C]formaldehyde (19). The HPLC-purified peptide had a specific activity of 1.5 mCi/mmol (1 mCi = 37 MBq). Equilibrium dialysis was performed using microcells in the Spectrum equilibrium dialysis chamber. Con A (200 μl, 0.1 mg/ml) was placed in one chamber and peptide (200 μl) was placed in the other. The chambers were separated by a SpectraPor 4 membrane with a molecular weight cutoff of 12,000–14,000. Dialysis was performed for 20 hr at 4°C. Samples from each chamber were removed and 50-μl aliquots were combined with 5 ml of Ready Scint (Beckman) for measurement of radioactivity in a Beckman model LS 6000TA scintillation counter.

**Inhibition of Dextran Precipitation.** The ability of peptide to inhibit the precipitation of Con A was assayed in the dextran 1355 system (20). Briefly, the precipitation reactions were conducted in plastic vials (1.5 ml) containing 18 μg of Con A and various amounts (5–20 μg) of dextran B-1355S in 100 μl of phosphate-buffered saline containing 1 M NaCl. The reaction mixtures were incubated for 48 hr, centrifuged for 10 min at 10,000 × *g*, and washed three times with phosphate-buffered saline containing 1 M NaCl. The protein content of the precipitate in each vial was determined by the method of Lowry with bovine serum albumin as the standard. Stock solutions of the sugar standard methyl α-D-mannopyranoside and all peptides were prepared in phosphate-buffered saline or doubly distilled water. Inhibition of the precipitation was performed by addition of various amounts of sugar or peptide to the reaction mixture containing Con A (18 μg) and dextran B-1355S (15 μg).

## RESULTS

The peptide library was constructed as previously described (16) except that a variable octapeptide region was fused to the amino terminus of the phage coat protein pIII via a spacer peptide, ASGSA. This spacer is expected to reduce steric interference of the phage surface with bound receptor. The library, consisting of $1.4 \times 10^9$ independent phage recombinants, was screened in three cycles of panning, elution, and amplification against Con A immobilized either covalently on Sepharose beads or noncovalently on streptavidin-coated microtiter plates. Affinity purification was carried out in the presence of 2 mM $CaCl_2$ because saccharide binding by Con A is calcium-dependent (17). In one set of experiments, adherent phage were eluted with buffer containing methyl α-D-mannopyranoside or yeast mannan at concentrations sufficient to block rebinding of peptide at the sugar binding site. Increasing percentages of the input phage (0.039%, 6.7%, and 11%) were recovered in rounds 1, 2, and 3 of screening, suggesting that selective phage enrichment was occurring. In a second series of experiments, phage were eluted with a low-pH citrate buffer (pH 3.0) to denature the receptor–ligand interaction [note that at neutral pH Con A exists as a tetramer of identical 26.5-kDa subunits and undergoes reversible dissociation below pH 5.6 to give a dimeric species (21)]. Similar levels of phage recovery were also observed through three rounds of acid elution.

After the third round of panning, 19 of 19 randomly selected individual phage isolates eluted with carbohydrate and 14 of 15 phage eluted with citrate displayed specific binding to Con A as determined by ELISA (data not shown). DNA from specific binding clones was sequenced and the deduced peptide sequences are shown in Fig. 1. The peptides eluted with methyl α-D-mannopyranoside can be separated into three groups. The first contains eight different sequences, one of which appears in four separate phage clones examined. These peptides are grouped in Fig. 1*a* to show a distinctive consensus sequence Tyr-Pro-Tyr, with additional preferences for Val-Trp/Phe and Pro/Gly in the amino and carboxyl flanking sites, respectively. Note that the conserved motif is duplicated in the replicate clones. The second group includes two members that each contain a pair of cysteine residues separated by four intervening amino acids (raising the possibility of a conformational constraint imposed by a disulfide bond). There is no obvious homology among the third group of sequences, but they all appear very rich in charged or polar residues.

Phage recovered by low-pH elution include several identical clones and the majority bear a close resemblance to the first group of peptides eluted with methyl α-D-mannopyranoside (see Fig. 1*b*). The motif Tyr-Xaa-Tyr replaces the strict consensus Tyr-Pro-Tyr sequence from the previous experi-

| A | | B |
|---|---|---|
| YPYPVFVH | YNMYSTAA | YNYGWVEF |
| YPYYQYFM | AVNGCRHD | YNYGWVEF |
| YPYGSYWA | SYTHVASS | YNYGWVEF |
| VWYPYGAG | KGQAELLR | YNYGWVEF |
| VWYKYPGW | VFTDQKAQ | YNYGWVEF |
| DVFYPYPY | LNDNSAGY | YRYDIFRE |
| DVFYPYPY | WARNTSHS | YDYGSFSK |
| DVFYPYPY | | YSYPYYHL |
| DVFYPYPY | | NYDYMGIW |
| NRVWYPYG | | YPYAYIWT |
| | | VVFYDYGS |
| NCQGTACS | | YEAHYQYG |
| LRCGWGVC | | |
| | | GTWFTNFR |
| | | SRCGLLVE |

FIG. 1. Amino acid sequences (deduced from DNA sequence) of amino-terminal octapeptides of pIII from phage eluted with 200 mM methyl α-D-mannopyranoside (*A*) or with citrate buffer (pH 3.0) (*B*).

ment, though this latter triplet does occur in two of the acid-eluted clones. In several clones the amino acid substitutions at the flanking sites follow the preferences noted above. Two phage clones with no clear homology to previously observed sequences were also found. The binding site(s) on Con A for the peptide sequences we have isolated that do not conform to the consensus motif are unknown.

The peptide DVFYPYPYASGS was chemically synthesized and its binding interaction with Con A was evaluated in two separate assays. This sequence was selected because it contained a tandem repeat of the consensus motif from the mannoside elutions and appeared in multiple phage isolates after three rounds of panning. The tetrapeptide ASGS (from the spacer region of the pIII fusion protein) was appended to the octamer sequence to enhance its solubility. The ability of this dodecapeptide to displace a chromogenic ligand, *p*-nitrophenyl α-D-mannopyranoside from Con A was determined by UV difference spectrophotometric assay (18). Fig. 2*A* shows the effect of peptide concentration on receptor occupancy by *p*-nitrophenyl α-D-mannopyranoside at two fixed concentrations of the chromogenic sugar. The data from these two experiments can be fitted by the same line giving a $K_d$ of 46 μM for DVFYPYPYASGS at 20°C (Fig. 2*B*). This compares with a $K_d$ of 89 μM for methyl α-D-mannopyranoside under the same experimental conditions [literature values of 120 μM and 67 μM for methyl and *p*-nitrophenyl α-D-mannopyranosides, respectively, have been reported with this assay at 27°C (18)]. While displacement of the chromogenic sugar from Con A by methyl α-D-mannopyranoside was complete within seconds of mixing, binding of the dodecapeptide required as long as 90 min to attain equilibrium. The reason for this slow approach to equilibrium has not been determined. Reversible binding of the peptide by Con A was confirmed by separating the peptide from the lectin by passage through a dialysis membrane with a 10-kDa cutoff.

The peptide DVFYPYPYASGS was N-methylated with [$^{14}$C]formaldehyde and its affinity for Con A was determined by equilibrium dialysis at 4°C. Scatchard analysis (Fig. 3) showed the peptide to bind at a stoichiometry of ≈1 per Con A subunit, with a $K_d$ of 70 μM, in reasonable agreement with the UV difference data at room temperature. So and Goldstein (22) measured a $K_d$ of 48 μM at 2°C for methyl α-D-mannopyranoside by equilibrium dialysis.

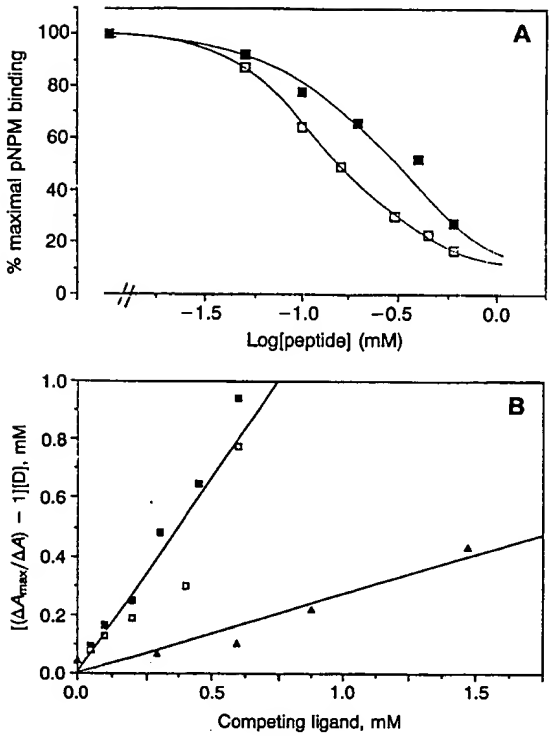


FIG. 2. (*A*) Effect of concentration of peptide DVFYPYPYASGS on binding of *p*-nitrophenyl α-D-mannopyranoside (pNPM) at 0.1 mM (□) or 0.2 mM (■) to Con A (27 μM binding sites). (*B*) Determination of dissociation constants ($K_d$) of peptide ligands for Con A by competition with pNPM. ■, DVFYPYPYASGS, 0.1 mM pNPM; □, DVFYPYPYASGS, 0.2 mM pNPM; ▲, RVWYPYGSYLTASGS, 0.1 mM pNPM. See *Materials and Methods* for explanation of ordinate label.

The octapeptide YRYDIFRE was selected as a representative Tyr-Xaa-Tyr-containing sequence from the clones eluted at low pH and was chemically synthesized. At concentrations as high as 10 mM, this peptide was unable to inhibit binding of *p*-nitrophenyl α-D-mannopyranoside to Con A (data not shown). Similarly, the tripeptide carboxamides YPY and YNY did not displace bound chromogenic sugar at 10 mM. To determine the ability of the consensus Val-(Trp/Phe)-Tyr-Pro-Tyr-(Pro/Gly) motif to confer Con A binding

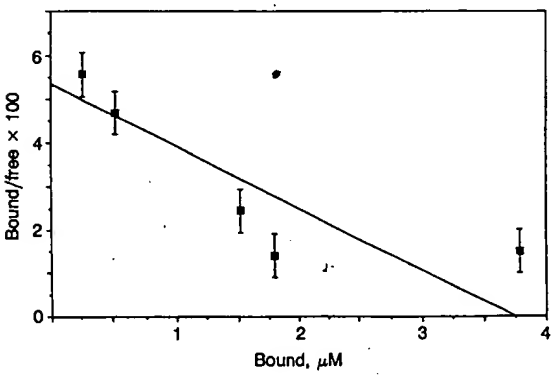


FIG. 3. Scatchard analysis of binding of N-[$^{14}$C]methylated DVFYPYPYASGS to Con A determined by equilibrium dialysis.

on a longer peptide, we tested the pentadecapeptide RVW-YPYGSYLTASGS in the spectrophotometric assay. This peptide inhibited sugar binding with an apparent $K_d$ of 230 $\mu$M, higher than that of the dodecapeptide (Fig. 2*B*).

Precipitation of polysaccharides and glycoconjugates by lectins has been extensively documented (23). Branched polysaccharides containing multiple terminal $\alpha$-glucopyranosyl, $\alpha$-mannopyranosyl, or fructofuranosyl groups are precipitated by Con A. In Fig. 4 the ability of peptides DVFY-PYPYASGS and RVWYPYGSYLTASGS to block precipitation of the $\alpha$-glucan dextran 1355 by Con A is contrasted with inhibition by methyl $\alpha$-D-glycosides. For the dodecapeptide an $IC_{50}$ value of 2.6 mM and for the pentadecapeptide an $IC_{30}$ of 5 mM compare with $IC_{50}$ values of 0.35 mM and 1.20 mM for methyl $\alpha$-D-mannopyranoside and methyl $\alpha$-D-glucopyranoside, respectively. Neither the octapeptides YRYDIFRE and YNYGWEVF nor the tripeptide carboxamide YPY displayed any inhibitory effects at 8 mM on dextran precipitation.

## DISCUSSION

A more detailed understanding of the molecular nature of protein–saccharide interactions should facilitate the design of inhibitors of carbohydrate-binding proteins and could influence the development of new therapeutic agents. For example, inhibitors of neuroaminidases or hemagglutinins might be useful in controlling viral infections, while antagonists of the selectin receptors ELAM1 or CD62 could prove effective in controlling inflammation and tissue damage (24). Although an endogeneous ligand, sialyl-Lewis X ($SLe^x$) [NeuAc$\alpha$2-3Gal$\beta$1-4(Fuc$\alpha$1-3)GlcNAc], for ELAM1 and CD62 has been synthesized (25–27), the synthesis of a series of carbohydrate analogues (28) is considerably more difficult than, for example, the synthesis of a series of analogues of a peptide hormone or protease substrate. Consequently, the identification of peptide antagonists of carbohydrate–receptor interactions should considerably simplify the synthesis of more potent or selective inhibitors.

A number of naturally occurring proteinaceous inhibitors have been isolated that compete for the binding sites of carbohydrate-specific proteins. For example, the protein tendamistat binds the enzyme $\alpha$-amylase with a $K_d$ of 9 pM (29–31). Interaction of the tripeptide epitope Trp-Arg-Tyr of tendamistat with the carbohydrate binding site is thought to play a critical role in complex formation (29, 32). These studies suggested that peptides might be found that competitively inhibit other classes of carbohydrate-binding proteins such as lectins or viral receptors. To test this notion we have screened a large library of peptide sequences expressed on the surface of filamentous phage for their ability to bind the lectin Con A.

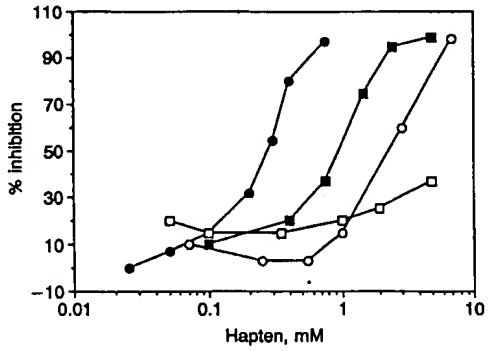


FIG. 4. Inhibition of the precipitation reaction between Con A and dextran B-1355S with methyl $\alpha$-D-mannopyranoside (●), methyl $\alpha$-D-glucopyranoside (■), and the synthetic peptides DVFYPYP-YASGS (○) and RVWYPYGSYLTASGS (□) as haptens.

An amino-terminal library of random octapeptides consisting of $\approx 1.4 \times 10^9$ members ($\approx$5% of all possible octamers) was affinity-purified against immobilized Con A. When phage were eluted by a low-pH citrate buffer to dissociate the phage–Con A complex, the sequence Tyr-Xaa-Tyr (where Xaa is predominantly a hydrophilic amino acid) was found in 12 of 14 clones recovered. When the adherent phage were repeatedly eluted with buffer containing methyl $\alpha$-D-mannopyranoside at a concentration sufficient to saturate the carbohydrate binding sites, proline was frequently observed in this triplet sequence. An additional preference for incorporation of Val-Trp/Phe and Pro/Gly residues at the sites flanking the triplet sequence was also apparent. A search of the GenBank database (January 1992) did not identify any known saccharide-binding proteins having a high degree of homology with this consensus sequence, although a highly homologous sequence (VWYPFPY) was found in the fimbrial assembly gene product (FimB) of several strains of the Gram-negative species *Bacteroides nodosus* (33). The fimbrial subunit proteins (*fimA* gene products) of many bacteria have lectin-like properties and mediate adhesion to eukaryotic cells through carbohydrate structures on the extracellular surface of the membrane. Interestingly, the cell-surface receptors for many bacteria are mannose-containing oligosaccharides (34). While adherence by the organism *B. nodosus* is not believed to be mannose-dependent, the structure of its eukaryotic receptor is unknown (33).

Two peptides containing the motif Val-(Trp/Phe)-Tyr-Pro-Tyr-(Pro/Gly) were synthesized and assayed for their ability to inhibit binding by Con A of known monosaccharide and polysaccharide ligands. The $K_d$ values for the dodecapeptide DVFYPYPYASGS were determined to be 46 $\mu$M and 70 $\mu$M, when measured by UV difference spectroscopy (20°C) and equilibrium dialysis (4°C), respectively. These values compare closely with the binding affinities of two monosaccharide ligands, methyl and *p*-nitrophenyl $\alpha$-D-mannopyranosides [$K_d$ of 120 and 67 $\mu$M at 27°C (18)]. The affinity of the pentadecapeptide RVWYPYGSYLTASGS was lower by a factor of $\approx$5 ($K_d$ of 230 $\mu$M). These peptides also inhibited Con A-dependent precipitation of dextran 1355 with $IC_{50}$ values of 2.6 mM and $\approx$15 mM, respectively. The higher inhibitory constants found in the precipitation assay presumably result from the multivalency of the lectin–dextran interaction. However, the 5-fold difference in affinity of the 12-mer and 15-mer peptides for Con A correlates well with the difference in their respective $IC_{50}$ values in the precipitation assay.

Although a number of hydrophobic binding sites exist on the Con A surface, including sites for fluorescent dansyl derivatives (35, 36), tryptophan or indoleacetic acid (37), and benzoic acid derivatives (38), a number of studies indicate that the chromogenic ligand *p*-nitrophenyl $\alpha$-D-mannopyranoside binds at the saccharide binding site (18, 39, 40). The fact that the potency of the dodecapeptide in the UV difference assay is dependent on the concentration of the chromogenic mannoside, and that the peptide binds with a stoichiometry of $\approx$1 per Con A subunit and also inhibits dextran precipitation, suggests that this peptide also binds at the saccharide binding site (or an overlapping site). A less likely alternative is that this ligand binds a second site, whose occupation induces a conformational change in the protein that abolishes binding of saccharides to Con A.

The saccharide binding site of each Con A subunit has been shown by X-ray crystallography to be a shallow crevice near the protein surface (41). Hydrogen bonds and van der Waals interactions are the predominant forces involved in binding of methyl $\alpha$-D-mannopyranoside, as is the case with most carbohydrate-binding proteins (1, 42). The sugar interacts with

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# Random Peptide Libraries: A Source of Specific Protein Binding Molecules

JAMES J. DEVLIN,* LUCY C. PANGANIBAN, PATRICIA E. DEVLIN

**Libraries of random peptide sequences were constructed and screened to identify peptides that specifically bind to proteins. In one of these about $2 \times 10^7$ different 15-residue peptide sequences were expressed on the surface of the coliphage M13. Each phage encoded a single random sequence and expressesd it as a fusion complex with pIII, a minor coat protein present at five molecules per phage. Phage encoding nine different streptavidin-binding peptide sequences were isolated from this library. The core consensus sequence was His-Pro-Gln and binding of these phage to streptavidin was inhibited by biotin. This type of library makes it possible to identify peptides that bind to proteins (or other macromolecules) that have no previously known affinity for peptides.**

SMALL MOLECULES THAT INTERACT with proteins (enzyme inhibitors or receptor agonists and antagonists, for example) are used in research and in clinical therapy. These small molecules can be developed by rational design or isolated by screening large numbers of naturally occurring or synthetic compounds. Frequently there is not enough information available for design, and assembling and screening a large library of compounds has been time consuming and expensive. We now describe the use of biological expression systems to facilitate both the production of large libraries of random peptide sequences and the screening of these libraries for peptide sequences that bind to particular proteins.

A major goal has been to increase the number of sequences that can be screened at one time. We produced the libraries by cloning synthetic DNA that encoded random peptide sequences into *Escherichia coli* expression vectors. For our first library, we used the phage λgt11 (*1*) to express $4 \times 10^6$ random 15-residue peptide sequences as fusion proteins with β-galactosidase. Although we identified fusion proteins that were recognized by an antibody to a linear epitope of the feline leukemia virus, this system was limited to screening about $10^6$ peptide sequences at a time. To increase the number of sequences that could be screened by potentially several orders of magnitude, we then investigated a filamentous phage expression system.

In the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage (*2*), thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at concentrations well over $10^{13}$ phage per milliliter, large numbers of phage can be screened at one time. Second, since each infectious phage encodes the random sequence expressed on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd, and f1 have five copies of a minor coat protein, pIII, on the surface (*3*). Parmley and Smith have shown that several foreign epitopes can be expressed at the $NH_2$-terminal end of pIII and furthermore, phage bearing one of these epitopes could be recovered from a large excess of phage lacking this epitope (*4*). They also suggested that if it were possible to express a large number of random peptide sequences, this system could be used to characterize the epitopes recognized by antibodies.

We have produced a large library of phage expressing random 15-residue peptide sequences as gene III fusion proteins; the accompanying article by Scott and Smith (*5*) describes a library of random six-residue sequences also expressed as pIII fusion proteins. We constructed the new expression vector, M13LP67, by making noncoding base changes to introduce restriction enzyme sites into gene III of M13mp19 and by placing a β-lactamase gene in the polylinker 3′ of the *lac* promoter (*6*). Ligation of complimentary oligonucleotides into the new sites in gene III led to mature pIII fusion proteins with the predicted $NH_2$-terminal sequence of Ala-Glu-$Xxx_{15}$-$Pro_6$-Ala-Glu (where Xxx represents random amino acid residues) (*7*). We retained the first two residues of the wild-type mature $NH_2$-terminus (Ala-Glu) because they may influence processing by signal peptidase, which is required to produce infectious phage (*8*). Both the frequency of termination codons and the variation in the number of codons for each amino acid residue was reduced by using $(NNS)_{15}$ to encode the 15 random residues (where N is a mixture of G, A, T, and C and S is a mixture of G and C). NNS encodes all 20 amino acids but will produce only one of the three termination codons. In the portion of the oligonucleotide complementary to $(NNS)_{15}$, we used deoxyinosine because of its relatively unselective base pairing ability (*9*). Polyproline tends to adopt an extended structure (*10*); thus, the random residues were followed by a spacer consisting of six proline residues, which was inserted to make the random peptides more accessible by moving them away from the rest of the protein. Since pIII function is required to infect *E. coli* (*11*), the Ala-Glu sequence was repeated after the proline linker to retain the entire uninterrupted sequence of pIII.

After these oligonucleotides were ligated into the M13LP67 expression vector, DNA was introduced into *E. coli* by electroporation, and these cells were plated with fresh *E. coli* cells (*12*). Successful introduction of the vector into a host cell resulted in a plaque of slow-growing phage-producing cells. Modification of pIII may reduce infectivity (*4*), and hence some phage replicating in a competitive environment may become underrepresented in the library. Such competition among the phage was avoided by plating transfected (by electroporation) cells at a density such that the plaques just touched one another (about 400 plaques per square centimeter).

After harvesting phage from $2.8 \times 10^7$ plaques, we determined what percentage of phage had the potential to express random peptide sequences. Phage that encoded random sequences contained an extra 69 base pairs (bp) in the 5′ end of gene III. Using polymerase chain reaction (PCR) to amplify the DNA from this region, we found that the 69-bp insert was present in 71 percent of the phage (*13*). Thus the plate stocks from the $2.8 \times 10^7$ original plaques contained $\sim 2 \times 10^7$ independent phage that expressed random sequences. Since there are $3 \times 10^{19}$ different possible 15-residue peptides, any given sequence has only one chance in $2 \times 10^{12}$ of occurring more than once in the library, assuming that the synthesis and resolution of the mismatched oligonucleotides is random.

We then addressed the issue of whether proteins other than antibodies to epitopes of linear peptides are able to bind to some random peptide. We screened the library for phage that bound to the biotin-binding protein, streptavidin, a protein with no known affinity for peptides. Furthermore, since one method to identify protein-binding peptides would be to screen the library with biotinylated proteins and then isolate protein-phage complexes on streptavidin-

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EXHIBIT A

coated surfaces, it would be necessary to know whether phage in the library would bind directly to streptavidin.

We used streptavidin-coated polystyrene plates as an affinity matrix for examining the phage stock. Expecting a $10^3$ to $10^4$ enrichment during each round of selection (*4*) and since the library had a complexity of $2 \times 10^7$, we performed two rounds of selection. In the initial selection, $10^{12}$ phage were adsorbed to a 60-mm plate. The plates were coated with streptavidin, bovine serum albumin was then added, the phage were adsorbed for 10 min, and the plates were washed and eluted (*4*), yielding $4 \times 10^5$ phage. After preparing a plate stock (*14*) to amplify the eluted phage, we repeated the adsorption and elution from a streptavidin-coated plate, starting with $10^{10}$ phage and eluting $10^8$ phage. We plated these phage at low density and prepared individual phage stocks from 60 randomly selected plaques (*14*).

We then determined whether the phage from these 60 isolates specifically bound to streptavidin. To avoid problems in interpreting background binding to streptavidin-coated plates, we mixed the individual phage preparations with M13mp19 phage and tested streptavidin coated plates for ability to enrich the peptide-expressing phage relative to the M13mp19 phage in the mixture. We compared the ratio of peptide-expressing phage to M13mp19 both in the initial mixture and in the eluate from the streptavidin plate.

Adsorption to and elution from streptavidin-coated plates enriched 56 of the 60 isolates by a factor of 10 relative to M13mp19. The DNA sequence analysis of 20 of the enriched isolates predicted nine different random peptide sequences, some of which occurred more than once in the 20 isolates. As examples of the nine different phage, the enrichment data for isolates A to I are shown in Table 1. For comparison, the data are shown for two isolates that failed to show enrichment and for M13LP67. If the plates were only blocked with BSA, but not coated with streptavidin, no enrichment occurred. For those phage tested, biotin (1 μM) significantly reduced the enrichment (Table 1).

Alignment of the His-Pro sequence found in the nine different predicted peptide sequences revealed an obvious consensus sequence (Table 2). The location of the consensus sequence in isolate B suggests that even the region of the random peptide closest to the proline linker is accessible for binding to foreign proteins. We have also sequenced the inserts from six random isolates that had not been selected for streptavidin binding; these predicted the expected distribution of amino acid residues and none of these predicted a His-Pro sequence. Although both histidine and biotin comprise a nitrogen and carbon containing ring, the exact nature of the interaction with streptavidin remains to be elucidated.

**Table 2.** The predicted sequences of the random peptides expressed by 20 streptavidin binding phage isolates. The sequences have been aligned on the common His-Pro sequence and are given in single letter code (*15*). The number of times each sequence occurred in the 20 isolates is given in the frequency column.

| Isolates | Frequency | Peptide sequences |
|---|---|---|
| A | 3 | SDDWWHD HPQN LRSS |
| B | 1 | MLWYSPHSFS HPQN T |
| C | 1 | SWWLSW HPQN TKELG |
| D | 5 | ISFENTWLW HPQF SS |
| E | 1 | LC HPQF PRCNLFRKV |
| F | 2 | PC HPQY RLCQRPLKQ |
| G | 2 | QPFL HPQG DERWYMI |
| H | 1 | ALCCLSSP HPNG AIF |
| I | 4 | LN HPMD NRLHVSTSP |
| *Consensus* | | HPQn |

**Table 1.** Enrichment of individual phage isolates on streptavidin-coated plates. For each isolate, 5 μl of phage stock (each stock had a different titer) and $5 \times 10^9$ M13mp19 phage were mixed in tris (50 mM, pH 7.5), 150 mM NaCl, bovine serum albumin at 100 μg/ml, and (for the +Biotin column) 1 μM biotin; the final volume was 50 μl. The phage from 30 μl of each mixture were then adsorbed to and eluted from streptavidin-coated 96-well plates (*4*). A reduction in the number of M13mp19 phage (blue plaques) per isolate phage (white plaques) indicated enrichment and was determined by plating both the initial mixtures and the eluates on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Xgal) indicator plates (*1, 6*). Phage from isolates A to I were reproducibly enriched (by a factor of $10^3$ to $10^5$); however, this is a qualitative assay and the significance of the differences in enrichment among the isolates A to I is not apparent. Results shown without biotin represent one of five separate experiments, results with biotin represent one of two experiments.

| Isolate | M13mp19 plaques per isolate plaque | | | | | |
|---|---|---|---|---|---|---|
| | − Biotin | | | + Biotin | | |
| | Initial mixture | Eluate | Enrichment | Initial mixture | Eluate | Enrichment |
| A | 28 | 0.002 | $1.4 \times 10^4$ | | | |
| B | 58 | 0.003 | $1.9 \times 10^4$ | 25 | 2 | 13 |
| C | 19 | 0.0007 | $2.6 \times 10^4$ | | | |
| D | 60 | 0.0009 | $6.7 \times 10^4$ | 320 | 3 | 107 |
| E | 140 | 0.012 | $1.2 \times 10^4$ | 110 | 1 | 110 |
| F | 23 | 0.0091 | $2.5 \times 10^3$ | | | |
| G | 28 | 0.0009 | $3.1 \times 10^4$ | 21 | 2 | 10 |
| H | 26 | 0.0024 | $1.1 \times 10^4$ | 48 | 1 | 48 |
| I | 16 | 0.002 | $8.0 \times 10^3$ | 17 | 6 | 2.8 |
| Y | 11 | 5 | 2.2 | | | |
| Z | 15 | 4 | 3.8 | | | |
| M13LP67 | 9 | 5 | 1.8 | | | |

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6. By in vitro mutagenesis, the sequence CCGCTG starting at position 1631 in M13mp19 was converted to CGGCCG, thereby producing an Eag I restriction enzyme site without changing the amino acids encoded in this region (*1*). Similarly, the sequence TGTTCC at position 1611 was converted to GGTACC to produce a Kpn I site. A β-lactamase gene was placed in the polylinker to allow selection while propagating the vector as a plasmid; however, it also disrupted the expression of the β-galactosidase α peptide, resulting in the production of white plaques on Xgal plates (*1*). The β-lactamase gene was obtained by PCR (*16*) amplification with the plasmid pUC19 as the template with the following two oligonucleotides as primers:
(i) GCTGCCCGAGAGATCTGTATATATGAGTAAACTTGG
(ii) GCAGGCTCGGGAATTCGGGAAATGTGCGCGGAACCC.
The PCR product was digested with Bgl II and Eco RI (these sites are underlined in the primers) while double-stranded, replicative form of the phage DNA was digested with Bam HI and Eco RI. The appropriate fragments were gel-purified, ligated, and transformed into *E. coli*; cells harboring phage with the appropriate insert were selected on ampicillin plates (*1*).
7. M13LP67 DNA, which had been digested with Eag I and Kpn I, was ligated for 4 hours at room temperature and then overnight at 15°C to these two oligonucleotides (X = deoxyinosine):
(i) CTTTCTATTCTCACTCCGCTGAA(NNS)$_{15}$-CCGCCTCCACCTCCACC
(ii) GGCCGGTGGAGGTGGAGGCGG(XXX)$_{15}$-TTCAGCGGAGTGAGAATAGAAAGGTAC
The oligonucleotides were first mixed together and heated to 95°C for 5 minutes and then cooled to room temperature in 15-μl portions. The ligation mixture contained digested M13LP67 DNA (45 ng/μl), a fivefold molar excess of oligonucleotides, T4 ligase at 3.6 units/μl (New England Biolabs), 25 mM tris, pH 7.8, 10 mM $MgCl_2$, 2 mM dithiothrei-

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12. H249 cells (a *recA*, *sup*°, F′ derivative of MM294) were prepared for transfection by electroporation (*17*). For electroporations, 85 μl of cells (approximately $4 \times 10^9$) were mixed with 1 μg of M13LP67 DNA that had been resuspended in 1 mM Hepes after ligation (*7*). This mixture was then subjected to a 560-V, 5-ms pulse in a 0.56-mm gap electrode with a BTX electroporation device. Immediately after electroporation, the contents of the electrode were mixed with freshly cultured cells and plated (*1*).
13. Phage from 40 plaques were transferred by toothpick to tubes containing all the components necessary for PCR (*16*) including these two oligonucleotides:
(i) TCGAAAGCAAGCTGATAAACCG
(ii) ACAGACAGCCCTCATAGTTAGCG
After 40 cycles, the PCR products from phage with and without an insert (296 bp compared to 227 bp) could be easily distinguished on a 2 percent agarose gel.
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# Polarity and Velocity of Sliding Filaments: Control of Direction by Actin and of Speed by Myosin

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**Myosin filaments, which are responsible for a large repertoire of motile activities in muscle and nonmuscle cells, can translocate actin filaments both toward and away from their central bare zone. This bidirectional movement suggests that there is enough flexibility in the head portion of the tightly packed myosin molecules in the native myosin filaments to move actin filaments not only in the expected direction, but also in the direction opposite to that predicted by the regular structure of muscle—away from the center of the myosin filament.**

MYOSIN FILAMENTS EXHIBIT A tight packing of the rod portion of the myosin molecules with the heads forming projections of opposite polarity on either side of a narrow central bare zone (*1*). It is commonly accepted that the cyclical interaction of myosin heads with polar actin filaments generates force that pulls the actin filaments toward the center of the bipolar myosin filament resulting in shortening of the muscle (*1*). Recently, an in vitro motility assay has been developed that allows for the visualization of the movement of fluorescently labeled actin filaments over a surface randomly coated with myosin (*2*, *3*). In this assay, the myosin molecules are not directly imaged and therefore their precise orientation is not known.

We have isolated large native thick filaments from clam adductor muscles with the use of a gentle technique (*4*). These myosin filaments can be directly visualized by video-enhanced differential interference contrast (DIC) microscopy (*5*) and appear as spindle-shaped filaments of lengths up to 20 μm (Fig. 1) (*6*). When bound to a glass surface, these myosin filaments translocated fluorescently labeled actin filaments in the in vitro motility assay (Fig. 2A). Direct comparison between the DIC and fluorescence images revealed that fluorescently labeled actin filaments could bind at any position on the myosin filament and commence directed movement (Fig. 2). Actin filaments moved both toward and away from the center of the myosin filament. Both long (2 to 8 μm) and short (<1 μm) actin filaments exhibited this behavior. The movement of actin filaments away from the center of the myosin filament is opposite that which normally occurs in muscle contraction. Analysis of the movement demonstrated that those actin filaments traveling toward the center of the myosin filament moved at a fast rate of $8.8 \pm 1.4$ μm/s, whereas those that were traveling away from the center moved at the much slower rate of $1.0 \pm 0.3$ μm/s (Fig. 2B). Some actin filaments traveled the entire length of the thick filament. In these cases the actin filament would bind to a myosin filament and commence moving at the fast rate until it crossed the bare zone, where it would slow abruptly upon encountering myosin heads of the opposite polarity. A single actin filament could reverse direction of travel by detaching and rapidly reattaching after undergoing a 180° or 360° end-to-end flip (Fig. 3A). This occurred most frequently with short actin filaments, which undergo rapid Brownian movements upon detachment. The direction of travel after the flip was correspondingly recovered or reversed, consistent with the intrinsic polarity of the actin filament. On some occasions a longer actin filament sliding off the end of the myosin filament reattached through its initial leading end and proceeded to move back down the same myosin filament in the opposite direction. Two actin filaments traveling in opposite directions on a single myosin filament could pass one another unimpeded, which is not surprising given the relatively large diameters of the myosin filaments (Fig. 3B). When this occurred one filament would be traveling at the fast speed and the other at the slower speed.

We also observed that the actin filament was very flexible and behaved more as a rope than as a rigid rod when moving in this assay. When a long actin filament traveling at the fast rate crossed into the region of opposite polarity of the myosin filament, its leading end slowed abruptly while the tail end, which was still in contact with myosin heads of the correct polarity, continued to move fast. Because of the difference in the sliding speeds of each end of the actin filament, it formed a flexible loop in the middle portion that was over the bare zone and was free of attachment (Fig. 3C). Actin filaments could also interact simultaneously with more than one adjacent myosin filament, producing independent sliding actions and often undergoing large changes in angles (Fig. 3D). The implications of this flexibility may be more commonly manifest in nonmuscle systems where the actin filaments are typically much longer than the myosin filaments and where a single actin filament may possibly interact with more than one myosin filament (*7*). This flexible nature of the long actin filaments indicates that the actions of well-separated myosin heads are not necessarily integrated along the actin polymer chain. Actin filaments often exist in bundles within cells. Such bundles may allow for a structure with more

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# A Novel PCNA-Binding Motif Identified by the Panning of a Random Peptide Display Library†

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ABSTRACT: Proliferating cell nuclear antigen (PCNA) has recently been identified as a target for the binding of proteins involved in DNA replication, DNA repair, and cell cycle control. The interactions between PCNA and a number of these proteins are known to be mediated by a conserved peptide motif. In this study, a random peptide library in which peptide sequences are displayed on the *E. coli* bacterial flagellin protein was screened for PCNA-binding sequences. Analysis of the retrieved peptide sequences verified the presence of the known PCNA-binding motif. In addition, a second, larger group of peptides containing a different consensus sequence for PCNA binding was discovered. This sequence was found to be present on DNA polymerase δ, and a peptide conforming to this sequence was demonstrated to bind to PCNA. Database search and analysis show that many proteins contain the second consensus sequence. These include proteins that are involved in DNA replication, repair, and cell cycle control. The demonstration of this second PCNA-binding motif may provide a basis for identifying and experimentally testing specific proteins for the structural basis for PCNA binding.

Proliferating cell nuclear antigen (PCNA) is a highly conserved eukaryotic protein that functions in DNA replication as a molecular sliding clamp that permits highly processive synthesis by DNA polymerase δ (pol δ) (*1*). PCNA, originally identified as a processivity factor for pol δ, has been intensively investigated both in terms of its structure and in terms of its role in cellular processes. Expression of recombinant human PCNA and its physicochemical characterization established that it is a trimeric protein (*2*), and the crystal structures of both yeast PCNA (*3*) and human PCNA (*4*) have shown that they are structurally and functionally homologous to the T4 gene 45 protein and the β subunit of *E. coli* DNA polymerase III holoenzyme DNA sliding clamps (*5, 6*). PCNA interacts with the clamp loader replication factor C (RFC) (*7*−*9*), DNA polymerase δ (*10, 11*), replication endonuclease FEN-1 (*12, 13*), and DNA ligase I (*14*) and plays a role in both leading- and lagging-strand DNA synthesis at the replication fork. During the past several years, it has been shown that PCNA also interacts with proteins involved in cell cycle progression and DNA repair. The DNA repair endonuclease XPG (*15*), the major nuclear uracil DNA−glycosylase (UNG2) (*16*), and the mismatch repair protein MSH2−MSH3 heterodimer (*17*) interact with PCNA. PCNA also binds to DNA (cytosine-5) methyltransferase (MCMT) (*18*), cyclin D (*19*), the cell cycle regulated nuclear protein Gadd45 (*20, 21*), and the cell cycle regulatory protein p21 (*22, 23*). Thus, PCNA, by virtue of its functions as a sliding clamp, may have multiple cellular functions associated with processes involving DNA modification or synthesis. Moreover, it may be a target for cell cycle regulation, as evidenced by its interactions with cyclin D, p21, p57 (*24*), and GADD45 (*20, 21*).

The ability of PCNA to interact with multiple protein partners having disparate structures is explicable at least in part through the existence of a PCNA-binding motif that is present on a number of its binding proteins (*25*). This conserved PCNA-binding motif was termed the PCNA interaction protein box (PIP-box) (*26*). An alignment of these binding motifs shows that it consists of the sequence Q-x-x-(h)-x-x-(a)-(a) (where "h" represents residues with moderately hydrophobic side chains, e.g., L, I, M; "a" represents residues with highly hydrophobic, aromatic side chains, e.g., F, Y; and "x" is any residue) (*25, 26*). Proteins which exhibit such a PCNA-binding PIP-box include p21, Fen1, XPG, and Dacapo protein, which is a cyclin-dependent kinase inhibitor (*27, 28*), and the *Pogo* transposon (*29*). In the case of p21, the structural basis for the interaction of this motif with PCNA has been determined at the atomic level by crystallographic analysis of a p21 peptide−PCNA complex (*4*). The peptide consisted of residues 139-GRKRRQTSMTDFYHS-KRRLIFS-160, and harbors the sequence QTSMTDFY which conforms to the PIP-box. The interaction of the peptide with PCNA involves three general features: interaction with a large hydrophobic pocket, a small hydrophobic pocket, and an extended interaction with the interdomain connector loop of PCNA (*4*). The residues in the PIP-box interact with the large hydrophobic pocket, and involve the two aromatic residues of the PIP-box. Thus, in the case of p21, the PIP-box represents only part of the protein−protein interface with PCNA. A 20 amino acid sequence that exists in both the N-terminal region of DNA ligase I and the large subunit of

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RFC has been proposed to function as a replication factory targeting sequence (RFTS). This sequence also binds to PCNA (*30*).

Because PCNA appears to be involved in the binding of a number of protein partners, the question of whether it contains sites for recognizing other peptide motifs can be raised. In addition, the short sequence of the PIP-box, and the fact that only three of eight residues in this sequence are conserved, suggests that its presence in a given protein does not necessarily provide strong evidence that the protein binds PCNA. Conversely, not all the known PCNA-binding proteins contain sequences which might conform to the PIP-box motif, e.g., cyclin D and pol ϵ. Mutational analyses of PCNA have shown that mutations in different regions of PCNA can result in differential effects with a given partner [reviewed by Tsurimoto (*31*)]. To obtain more information on the peptide motifs that are recognized by PCNA, a random peptide library was screened against PCNA. The results provide evidence that PCNA may bind to a second class of peptides in addition to those conforming to the PIP-box, and further verify the existence of the conserved motif (PIP-box).

## EXPERIMENTAL PROCEDURES

*Materials.* The FliTrx random peptide library was obtained from Invitrogen (San Diego, CA). The Sephacryl S-200 column and Mono P column for PCNA purification, the activated CH-Sepharose, the T7 Sequenase version 2.0 DNA sequencing kit, the protein biotinylation system, and the ECL chemiluminescence detection reagents were purchased from Amersham−Pharmacia Biotech Inc. The QIA prep miniprep kit for plasmid isolation was obtained from QIAGEN. Pol δ synthetic peptides, C1 (1047-LEERFSRLWTQCQRCQGSLHED-1068), C2 (1069-VICTSRDCPIFYMRKKVRKDLED-1090), C3 (1091-DLEDQEQLLRRFGPPGPEAW-1107), N2 (129-GVTDEGFSVCCHIHGFAPYFY-149), N2a (129-GVTDEGFSVC-138), N2b (139-CHIHGFAPYFY-149), N2−1 (mutant of N2, 129-GVTDEGASVCCHIHGFAPYFY-149, F → A), N2−2 (mutant of N2, 129-GVTDEGFRVCCHIHGFAPYFY-149, S → R), N2−3 (mutant of N2, 129-GVTDEGFSVACHIHGFAPYFY-149, C → A), N2−4 (mutant of N2, 129-GVTDEGFSVCCAIHGFAPYFY-149, H → A), N2−5 (mutant of N2, 129-GVTDEGFSVCCHIAGFAPYFY-149, H → A), N2−6 (mutant of N2, 129-GVTDEGFSVAAHIHGFAPYFY-149, CC → AA), N2AAA (mutant of N2, 129-GVTDEGFSVCCHIHGFAPAAA-149, YFY → AAA), and N4 (276-RLKEKATQCQLEADVLWSDV-295), were synthesized by the Protein Chemistry Core Laboratories (Miami, FL). The IMC medium used for growth of the *E. coli* peptide library was 6 g/L $Na_2HPO_4$, 3 g/L $KH_2PO_4$, 0.5 g/L NaCl, 1 g/L $NH_4Cl$, pH 7.4, 0.2% casamino acids, 0.5% glucose, and 1 mM $MgCl_2$. TGD buffer consists of 50 mM Tris-HCl, 5% glycerol, 1 mM DTT, pH 7.8. The blocking solution consisted of 1% nonfat dry milk, 150 mM NaCl, 1% α-methyl mannoside, and 100 μg/mL ampicillin in IMC medium. The 1 L of RM medium contained 6 g of $Na_2HPO_4$, 3 g of $KH_2PO_4$, 0.5 g of NaCl, 1 g of $NH_4Cl$, pH 7.4, 20 g of casamino acids, 10 mL of 50% glycerol, 100 μg/mL ampicillin, and 1 mM $MgCl_2$. The plating medium for RMG plates contained 15 g of agar in 1 L of RM medium containing 100 μg/mL ampicillin. TGEED buffer consists of 50 mM Tris-HCl (pH 7.8), 5% glycerol 0.5 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol.

*Preparation of PCNA.* Five milliliter overnight cell cultures of *E. coli* DHα containing the PCNA expression plasmid previously described (*2*) were used to inoculate 1 L cultures (Terrific media) and grown at 37 °C until the $A_{600}$ reached 0.3. After addition of isopropyl-1-thio-β-D-galactopyranoside to a concentration of 0.3 mM, the cultures were grown for another 16 h at 28 °C. The cells were harvested, and the PCNA were purified as described by Zhang et al. with minor modifications (*2*). Before loading on a Sephacryl S-200 column for purification, the preparation was first purified by chromatography on a Mono P column.

*Growth of the E. coli Peptide Library.* The FliTrx random peptide library is based on the system described by Lu et al. (*32*). The *E. coli* strain GI826 containing pFliTrx with inserts was grown with shaking for 18 h at 25 °C in IMC medium containing 100 μg/mL ampicillin. The cultures ($1 \times 10^{10}$ cells) in 50 mL of IMC medium were induced to express the thioredoxin−flagellin fusion proteins containing the peptide inserts by adding ampicillin (100 μg/mL) and tryptophan (100 μg/mL) and grown for another 6−8 h at 25 °C.

*Immobilization of PCNA on Culture Plates.* The purified PCNA was dialyzed overnight with TGD buffer. PCNA was immobilized on 60 mm plastic Petri dishes by adsorption from 1 mL of PCNA solution (100−120 μg of PCNA/plate) with gentle agitation for 1 h. After washing the plate with 10 mL of sterile water, the immobilized plate was agitated for 1 h with 10 mL of blocking solution.

*Panning of the Random Peptide Display Library.* The panning technique was performed as described in the manufacturer's procedure with minor modifications. After the 6 h induction, the following were added to the 50 mL of induced cells: 0.5 g of nonfat dry milk, 1.5 mL of 5 M NaCl, and 2.5 mL of 20% α-methyl mannoside. The induced cells (10 mL) were added to the plate containing immobilized PCNA. The plate was rotated gently for 1 min at 50 rpm and allowed to incubate for 60 min at room temperature. The bacterial culture was decanted. The plate was washed by gentle agitation for 5 min with 10 mL of IMC medium containing 100 μg/mL ampicillin and 1% α-methyl mannoside, and the wash was repeated 4 times. The fifth wash was fully decanted, and the PCNA-bound bacteria were detached into a small volume of IMC medium by vortexing the plate for 30 s. The detached bacteria were grown as described above. The culture was then induced by growth with tryptophan-containing medium, and the cycle of panning was repeated. Nine rounds of panning were performed.

*Biotin-Labeled PCNA.* Purified recombinant PCNA (1 mg/mL) was dialyzed overnight in 40 mM sodium bicarbonate buffer, and concentrated on a Centricon-30 filter. PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester. One milliliter of PCNA solution (0.5 mg/mL) was incubated with 25 μL of biotinamidocaproate *N*-hydroxysuccinamide ester (5 mg/mL) at room temperature for 1 h with constant agitation. The reaction mixture was passed through a Sephadex G-25 column equilibrated with 5 mL of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA). The conjugated PCNA was diluted 1000-fold in PBS containing 1% BSA and 0.2% sodium azide, and stored at 4 °C.

*Preparation of Positive Clones for Analysis.* After the ninth panning, the cultures were streaked onto RMG-Amp plates,

and single colonies were selected. Each colony was inoculated into 4 mL of RM medium, and grown at 30 °C to saturation with shaking (245 rpm) for 20 h until the $A_{600}$ reached 2.0−3.0. The cultures (100 μL) were then inoculated with 3 mL of IMC medium containing 100 μg/mL ampicillin and 100 μg/mL tryptophan, and grown at 30 °C for 8 h until the cells reached mid-log phase (0.6 $OD_{600}$). The remainder of the noninduced cultures was saved at 4 °C for DNA isolations.

*Overlay Blotting with Biotinylated PCNA.* After induction, the cells were harvested and resuspended in 150 μL of SDS−PAGE sample buffer and heated at 100 °C for 5 min. Ten microliters of sample was loaded and subjected to electrophoresis on SDS−PAGE (10% acrylamide), and transferred to a nitrocellulose membrane. The blot was blocked with 5% nonfat dry milk in PBST (1 × PBS, plus 0.05% Tween 20) for 2 h at room temperature with shaking followed by four washes of PBST for 10 min each. The blot was then incubated with biotinylated PCNA (0.5 μg/mL) at 4 °C overnight or for 2 h at room temperature with shaking. The blot was washed 5 times with PBST for 15 min each. It was subsequently incubated with streptavidin−horseradish peroxidase conjugate diluted in PBST (1:10 000) for 1 h at room temperature with shaking. After washing 5 times with PBST for 15 min each, the blot was developed using a chemiluminescence method (ECL detection system, Amersham).

*DNA Sequencing.* The remaining noninduced cultures from above were collected, and the pFliTrx plasmids were isolated. DNA sequencing was performed by the dideoxy chain termination method using the T7 Sequenase version 2.0 DNA sequencing system (Amersham). The primer used for DNA sequencing was the FliTrx forward sequencing primer, 5′-ATTCACCTGACTGACGAC-3′.

*Sequence Analysis.* The amino acid sequences of inserted peptides were analyzed by the Motif program at the GenomeNet Database (Kyoto University and the University of Tokyo, Japan). The protein sequence database used for search was SWISSPROT. The query pattern entered in the search was written in PROSITE format.

*Dot Blot Analysis for the Interaction between Pol δ Synthetic Peptides and PCNA.* About 5 μg of each peptide was dotted onto a nitrocellulose membrane and air-dried for 45 min at room temperature. After blocking the membrane with 5% nonfat dry milk in TBST (1 × TBS, plus 0.05% Tween 20) for 2 h at room temperature with shaking, the membrane was visualized by following the same procedures as for the PCNA overlay described above.

*Preparation of Immobilized Peptides and Adsorption of PCNA on N4- or N2−6-Sepharose.* Activated CH-Sepharose (0.25 g) was suspended in cold 2 mM HCl for 15 min and then washed 6 times with ice-cold 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (separated by centrifugation, 3000 rpm, 8 s). The gel was then mixed with the peptide solution (N4 and N2−6, 2.5 mg in 1 mL of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), and the suspension was rotated end-over-end overnight at 4 °C. The gel was blocked by incubation with 1 mL of 0.2 M glycine, pH 8, for 20 h at 4 °C and then washed 6 times with 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 (separated by centrifugation, 3000 rpm, 8 s). Purified recombinant human PCNA (40 μL, 0.1 μg/μL) was mixed with 40 μL of N4 or N2−6 coupled to CH-Sepharose. The suspension was mixed at 37 °C for 1 h. The beads were then centrifuged (3000 rpm, 8 s) and washed 7 times with 0.1 M $NaHCO_3$, 0.6 M NaCl, pH 8.3. After the final wash, the beads were resuspended in 40 μL of SDS−PAGE sample buffer and boiled for 5 min. The supernatant was then taken directly for SDS−PAGE and Western blot analysis using anti-PCNA antibody (*33*). The same procedure was used for coupling of bovine serum albumin, which was used as a control.

*Enzyme Activity Assay.* DNA polymerase δ activity was assayed with poly(dA)/oligo(dT) as a template-primer and [$^3$H]dTTP as the nucleotide donor. First, increasing amounts (1 mg/mL) of various synthetic peptides were added to 5 μL of PCNA (0.1 mg/mL) in each tube. The reactions consisted of either 0, 1, 2, 4, or 8 μL of the peptide(s). Each reaction was brought to the same volume by adding TGEED buffer. Each volume was mixed and allowed to react at room temperature for 1 h. Then, 5 μL of DNA polymerase and 45 μL of poly(dA)/oligo(dT) were added to each reaction. The reaction mixtures were incubated at 37 °C for 30 min. The samples were spotted onto DE81 filter paper circles and placed under a lamp for 10 min. Filters were washed 3 times in 0.3 M ammonium formate for 10 min each time and once with 95% ethanol for 5 min. Pol δ activity was determined by DNA binding to the DE81 filters as described by Lee et al. (*34*).

## RESULTS

*Panning of the FliTrx Random Peptide Display Library Using PCNA.* The FliTrx random peptide display library used is based on the display of peptides on the flagella of *E. coli*. The FliTrx library has a diversity of $1.77 \times 10^8$ individual dodecapeptides; each is flanked by the sequences CPG and GPC at its N and C termini, respectively. These peptides are inserted into the active-site loop of thioredoxin, which is itself fused into the major flagellin protein of *E. coli*. After induction of the flagellin fusion protein in the cells, the peptides are thus displayed on the flagella. The screening consisted of consecutive rounds of panning on Petri dishes to which PCNA was immobilized. After nine rounds of panning (see Experimental Procedures), the plasmids from individual *E. coli* isolates were isolated and analyzed. To confirm that these isolates were indeed binding to PCNA, a PCNA overlay method was employed to detect thioredoxin−flagellin fusion proteins that bind to PCNA. This was done by SDS−PAGE of the *E. coli* proteins, followed by overlay blotting with biotin-conjugated PCNA. Figure 1 shows an example of this analysis in which eight isolates were tested. It can be seen that there are five isolates which show a positive overlay for PCNA binding to a protein of 68 kDa. The latter size is consistent with an expected size of the fusion protein since *E. coli* thioredoxin has a molecular mass of 12 kDa (*35*) and *E. coli* flagellin has a molecular mass of 60 kDa (*36*).

*Analysis of the Peptide Sequences Isolated by Panning of the FliTrx Library.* Eighty-five peptide inserts were obtained from the panning of the FliTrx library, and 49 of these were positive clones by the PCNA overlay method. Inspection of the insert peptide sequences of these positive clones shows that they did not fall into a single family. In Table 1, two groups of peptide sequences which clearly form two separate families are shown. The first of these (Group 1, Table 1) contained those sequences that matched or were similar to

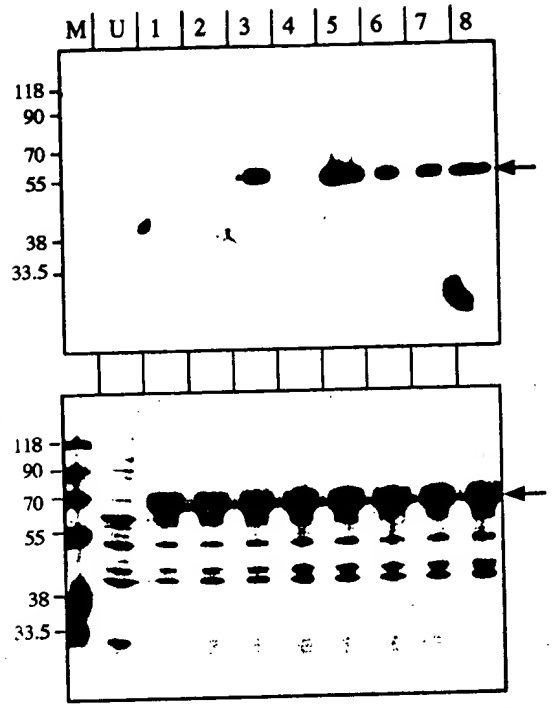


FIGURE 1: Binding of PCNA to thioredoxin−flagellin fusion proteins. The binding of PCNA to the thioredoxin−flagellin fusion proteins of the isolates obtained by panning of the random peptide library was tested by PCNA overlay of bacterial extracts (see Experimental Procedures). Induced cultures of isolated colonies were collected and resuspended in 150 $\mu$L of SDS−PAGE loading buffer. The samples were boiled for 5 min and then loaded onto SDS−PAGE (5 $\mu$L). After transfer to the nitrocellulose membrane, the blot was exposed to biotinylated PCNA and detected using streptavidin−horseradish peroxidase conjugate (see Experimental Procedures). The upper panel shows a representative overlay blot for eight individual isolates (lanes 1−8). The lane marked "M" contained the protein standards, and the lane marked "U" is that of a culture containing the FliTrx plasmid without induction. The lower panel shows the Coomassie blue stain for protein. The position of the 68 kDa thioredoxin−flagellin fusion protein is indicated by arrows.

the known PCNA PIP-box motif. Only the first five of the sequences in Group I can be obviously matched to the PIP-box. Sequences 8−6 and 9−15 conformed closely to the specifications of the PIP-box, Qxx(h)xx(a)(a) (h, residues with moderately hydrophobic side chains, e.g., L, I, M; a, residues with highly hydrophobic, aromatic side chains, e.g., F, Y; x, any residues). The next three possessed only a single aromatic residue, as has been found for the PIP-box for *Drosophila* p21 (*26*). The next two peptides (9−19 and 9−21) contained a pair of aromatic residues but without the conserved glutamine. PCNA overlay analysis confirmed that 9−19 reacts with PCNA. Members of the PIP-box motif which also do not contain the conserved glutamine are those present in p57 (*24*), and the PIP-box sequence that has been proposed to reside in the N2 region of the 125 kDa catalytic subunit of pol $\delta$ (*37*). Two additional sequences (7−3 and 7−2) are shown in Group I, but these can only be considered as marginal members of the PIP-box as they lack the aromatic residues.

The second group (Group II) only contained four members which are strongly related and are characterized by the presence of two or three aromatic residues. The search sequence Y-x(3)-[YT]-x(4)-W was used to search for related sequences in the SWISSPROT database using the PROSITE search engine. Nearly 1500 hits were obtained, and among the hits were a number of enzymes/proteins related to the replication of human viruses (not shown).

Table 1: Peptide Sequences from Screening of the FliTrx Random Peptide Library with PCNA as the Bait

| | |
|---|---|
| **Group I (PIP-Box motif)** | |
| 8-6 | K**Q**G R**L** A G **F F** K L G |
| 9-15 | F**Q** P A **V** G D **F Y** A S K |
| 7-1a | A T **Q** H S K R A **Y** A V G |
| 7-12 | S**Q** R R S G P **Y** A A T L |
| 9-47 | A C V N R **Q** S R E D E **F** |
| 9-19 | T T G S G H G **F Y** K P G |
| 9-21 | M W R S E **F Y** T P P D E |
| 7-3 | E F **Q** C P R E A L K A H |
| 7-2 | D F **Q** C P R E A L K A H |
| **Group II.** | |
| 8-2 | **Y** E G E **Y** I R S C **W** P G |
| 8-9b | **Y** E G E **Y** I R S C **W** P G |
| 8-9a | **Y** E G S T I R S C **W** P G |
| 8-9 | **Y** E G S T I P S C **W** P G |

*A New PCNA-Binding Motif Found from the Isolates of the FliTrx Random Peptide Library.* The third group of peptides that were aligned is shown in Table 2, and is subdivided into Groups IIIA, IIIB, and IIIC. This group consists of 29 members, about one-third of the total sequences that were isolated. Group IIIB contains 13 members, and is the largest of the 3 subgroups. Groups IIIA and IIIB are clearly related, while Group IIIC is more closely related to Group IIIB. The distinguishing feature of Groups IIIA and IIIB is the presence of a highly conserved KA pair, followed by two to four aliphatic hydrophobic residues that are mainly leucines. Group IIIA is distinguished from Groups IIIB and IIIC by the presence of a conserved pair of residues that consists of a basic residue often followed by leucine that is N-terminal to the KA pair that is absent in Groups IIIB and IIIC. Group IIIC is lacking the conserved basic residue of the KA pair that is present in Group IIIA and Group IIIB. The overall motif was termed the KA-box.

Attempts were made to search for related sequences using query patterns for the KA-box motifs in Groups IIIA and IIIB in the SWISSPROT database using the PROSITE search engine. However, because of the shortness of the sequence and a lack of very strong conservation, a large number of "hits" were obtained. For example, using a query pattern based on Group IIIA, over 600 hits were obtained using the PROSITE program. Using a shorter query pattern, based on Group IIIB, nearly 3000 hits were obtained. It is noted parenthetically that use of the PIP-box motif provides even larger numbers of hits. Included among the hits were a number of proteins that are involved with DNA replication, DNA repair, and cell cycle control. Among these were sequences present in the N-terminus of the catalytic subunit

Table 2: The Major Group of PCNA-Binding Peptides Isolated by Peptide Library Screening[a]

| | | |
|---|---|---|
| Group IIIA | 9-44 | HGLGAFQGREVI |
| | 9-36 | KIRHAGSGVGSL |
| | 9-47b | RLCKALNGPNES |
| | 9-47a | RLCKPANPPNES |
| | 9-47c | HLCKPLNGANES |
| | 9-26 | HLCKAHQWPVRE |
| | 9-47d | HLCKALMARTRV |
| Group IIIB | 9-13 | PGSGRAILHPWA |
| | 9-13a | PGSVRAIRHPWA |
| | 9-11 | LKALIAKGNFPS |
| | 9-59 | AGKASLITLRGR |
| | 9-43 | KARLGSLAIRCG |
| | 9-22 | KAGLGSLAIKCG |
| | 9-51 | MCGKAVLDLKLH |
| | 9-61 | RALDARLGAGGR |
| | 9-18 | AIRKAGQVTLIM |
| | 9-4 | ALIRVLRLQLGR |
| | 9-62 | LKPGLGVTAITL |
| | 9-56 | TEGKATNSRTLI |
| | 9-52 | LKAVEMLMGVLR |
| Group IIIC | 8-16: | TADSLLHLQNGK |
| | 8-7 | LALRRVKAISTV |
| | 7-1 | AHAALKRAYAVG |
| | 7-15 | AALAVSLMQCLR |
| | 9-28 | RGAVSVGMLAGS |
| | 8-21 | VSGCSLRQVCLL |
| | 8-8 | VAGGAGGRGALR |
| | 9-48 | ADVIVGLCVHAL |
| | 9-3 | VTWRLDVQLARP |

[a] Residues that are conserved over more than one group are shown in boldface and are shaded. Where a second residue in the same column is conserved, it is shown in boldface alone.

of pol δ. The region of similarity of the pol δ sequences falls in what is termed the N4 region by Yang et al. (*38*), and is conserved among pol δ enzymes from eukaryotes as well as in several viral polymerases including those of HSV, EBV, and CMV. An alignment of the N4 regions of pol δ of different species with members of the Group IIIA and IIIB peptides is shown in Table 3.

*The Peptide, N4, Containing the New PCNA-Binding Motif Interacts with PCNA.* In previous studies, we had examined the binding of PCNA to peptides conforming to conserved regions in the N-terminus of human pol δ, and have found that the N2 region, but not the N4 region, bound to PCNA by a dot blot procedure using a PCNA overlay method followed by detection with a monoclonal antibody against PCNA and a chemical staining procedure (*39*). For this reason, the issue of whether a peptide containing the N4 region would bind to PCNA was reinvestigated with the use of more sensitive detection methods. Dot blot experiments were performed in which samples of peptides were placed on nitrocellulose membranes and overlayed with biotinylated PCNA. The blot was visualized using a chemiluminescence method (see Experimental Procedures). A peptide corresponding to the N4 region of human pol δ p125 (276-RLKEKATQCQLEADVLWSDV-295) was tested together with peptides derived from other portions of the pol δ sequence (Figure 2). The peptides tested included three from the C-terminus (C1, C2, C3), and peptides based on the conserved N2 regions of pol δ. These were the N2 peptide (129-GVTDEGFSVCCHIHGFAPYFY-149), N2−6 (129-GVTDEGFSVAAHIHGFAPYFY-149), in which cysteines 138 and 139 were mutated to alanines and which has previously been shown to bind PCNA (*37, 39*), the two half-peptides of the N2 sequence, N2a (129-GVTDEGFSVC-138) and N2b (139-CHIHGFAPYFY-149), and N2AAA (129-GVTDEGFSVCCHIHGFAPAAA-149). Also included in these experiments were PCNA, purified calf thymus pol δ, and bovine serum albumin (BSA). The results showed that only peptides N4, N2, N2−6, and N2b gave positive signals (Figure 2). In the same experiment, positive signals were obtained with PCNA itself and with the pol δ enzyme but not with BSA. The difference between this experiment and our previous studies (*39*) is the use of a more sensitive chemiluminescence assay for binding, and suggests that the N4 peptide, while it does bind to PCNA, may not bind as strongly as the N2 peptide. It was also found that addition of the N4 peptide could successfully block the binding of biotinylated PCNA to the p125 catalytic subunit of pol δ in overlay experiments (data not shown).

A second experimental approach to confirm if these peptides could bind to PCNA was to test for their ability to inhibit the PCNA stimulation of pol δ activity. The results are shown in Figure 3. The N2 peptide, as a positive control, inhibits the PCNA stimulation of pol δ as we have previously shown (*39*). The N4 peptide also inhibited the PCNA stimulation of pol δ, as did the half-peptide N2b, although both of these were clearly less potent than the full-length N2 peptide. None of the other peptides tested (C1, C3, N2a, N2AAA) inhibited pol δ activity. The potency of inhibition of pol δ by N4 was roughly 5-fold less effective than the N2 peptide. These results demonstrate the ability of the N4 peptide to inhibit the PCNA stimulation of pol δ, consistent with its having the ability to bind to pol δ.

The demonstration that the C-terminal half, but not the N-terminal half of the N2 peptide, showed positive interactions with PCNA by inhibition assays provides additional evidence that the N2 peptide contains a variant of the PIP-box (*37, 39*). This is also consistent with the demonstration that the N2 peptide, but not the N2AAA peptide in which the aromatic resides of the PIP-box (*25, 26*) are mutated to alanine, is able to inhibit the PCNA stimulation of pol δ. Experiments in which other mutations of N2 (Figure 4) were tested showed that all these were able to inhibit pol δ activity, consistent with our previous data showing that these same peptides were able to bind biotinylated PCNA in overlay experiments (*37*).

A more direct test of the ability of the N4 peptide to bind PCNA was performed. The N4 and N2−6 peptides were immobilized on CH-Sepharose (see Experimental Procedures) and tested for their abilities to bind PCNA. Recombinant human PCNA was incubated with the peptide−Sepharose beads to which either N4 or N2−6 had been

Table 3: Alignment of the N4 Regions of the 125 kDa Subunit of Pol δ with the Group III Peptides[a]

| | | | | | |
|---|---|---|---|---|---|
| **N4 regions of pol δ:** | | | | | |
| soy-bean | | 237 | KTAKSLSYCQLEFDCL | 252 | AF020193 |
| *S. pombe* | | 260 | RYQNRVSNCQIEAWIN | 275 | L07734 |
| EBV | | 259 | RLQHRDSYAELEYDCE | 274 | V01555 |
| hamster | | 272 | RTEKKATQCQLEVDVL | 287 | AJ222691 |
| rat | | 272 | RAEKKATLCQLEVDVL | 287 | O54747 |
| bovine | | 275 | RPEGKATLCQLEADVL | 290 | M80395 |
| human | | 276 | RLKEKATQCQLEADVL | 291 | M01735 |
| **Peptides: IIIA** | 9-47b | | RLC-KALNGPNES | | |
| | 9-47a | | RLC-KPANPPNES | | |
| | 9-26 | | HLC-KAHQWPLRE | | |
| | 9-47d | | HLC-KALMARTRV | | |
| **Peptides: IIIB** | 9-59 | | AGKASLITLRGR | | |
| | 9-18 | | AIRKAGQVTLIM | | |
| | 9-43 | | KARLGSLAIRCG | | |
| | 9-61 | | RALDARLGAGGR | | |
| | 9-52 | | LKAVEMLMGVLR | | |
| | 9-56 | | TEGKATNSRTLI | | |
| | 9-4 | | AIIRVLRLQLGR | | |

[a] Sequences from the N4 regions of pol δ are aligned with representative peptides from Groups IIIA and IIIB. Conserved residues are shown in boldface and are shaded. Accession numbers are given on the right.

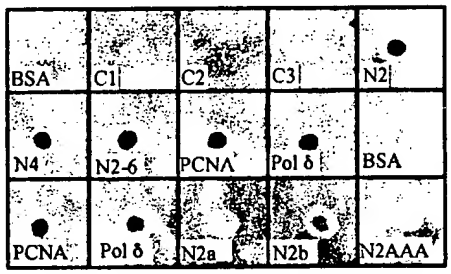


FIGURE 2: Dot blot analysis of the binding of PCNA to synthetic peptides. The synthetic peptides or proteins were dot blotted onto nitrocellulose and tested for PCNA binding by the use of biotinylated PCNA (Experimental Procedures). The blots were visualized using a chemiluminescence method. The synthetic peptides used were as follows: C1 (1047-LEERFSRLWTQCQRCQGSLHED-1068), C2 (1069-VICTSRDCPIFYMRKKVRKDLED-1090), C3 (1091-DLEDQEQLLRRFGPPGPEAW-1107), N2 (129-GVTDEGFSVCCHIHGFAPYFY-149), N2a (129-GVTDEGFSVC-138), N2b (139-CHIHGFAPYFY-149), N2−6 (mutant of N2, 129-GVTDEGFSVAAHIHGFAPYFY-149, CC → AA), N2AAA (mutant of N2, 129-GVTDEGFSVCCHIHGFAPAAA-149, YFY → AAA), and N4 (276-RLKEKATQCQLEADVLWSDV-295). BSA, purified calf thymus pol δ, and PCNA were also tested.

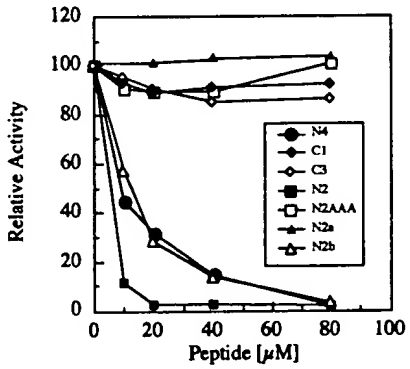


FIGURE 3: Inhibition of pol δ activity by the synthetic peptides N4 and N2. Human pol δ activity was assayed as described under Experimental Procedures in the presence of PCNA and varying concentrations (μM) of the peptides. Results are shown as relative activities. Pol δ synthetic peptides used in the assay include: C1 (1047-LEERFSRLWTQCQRCQGSLHED-1068), C3 (1091-DLEDQEQLLRRFGPPGPEAW-1107), N2 (129-GVTDEGFSVCCHIHGFAPYFY-149), N2a (129-GVTDEGFSVC-138), N2b (139-CHIHGFAPYFY-149), N4 (276-RLKEKATQCQLEADVLWSDV-295), and N2AAA (mutant of N2, 129-GVTDEGFSVCCHIHGFAPAAA-149, YFY → AAA).

attached; the beads were washed, extracted with SDS buffer, and then Western blotted with antibody against PCNA (see Experimental Procedures). The results show that both the N4-Sepharose and the N2−6-Sepharose beads were capable of binding PCNA as shown by SDS−PAGE and silver staining (Figure 5). Similar tests of bovine serum albumin coupled to Sepharose were negative.

Both experiments, dot blot and adsorption of PCNA on N4- or N2−6-Sepharose, show that peptides N4 and N2−6 are able to bind PCNA. Zhang et al. (*37*) recently found that the N2 region of DNA pol δ could interact with PCNA. The peptide N2−6 is similar to its parent peptide, N2, in the ability of PCNA binding. The ability of N2−6 to bind to PCNA further verifies that the C-terminal portion of the conserved region in the N-terminus of DNA polymerase δ is involved in PCNA binding.

## DISCUSSION

The results of this study show not only that sequences corresponding to the known PIP-box can be selected using a random peptide display library, but also that there is a novel family of peptides that bind to PCNA. This novel group comprised approximately one-third of the sequences isolated. While the alignment of these peptides did not provide a singularly tight consensus, the results reveal a novel peptide

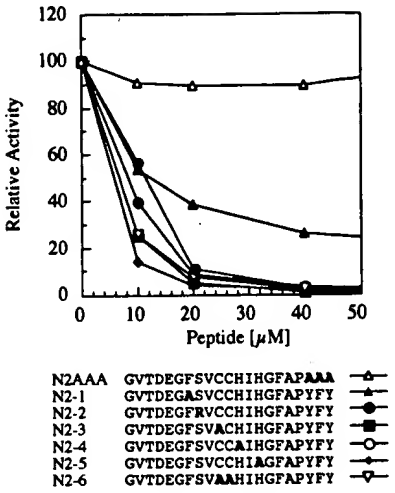


FIGURE 4: Inhibition of pol δ activity by the mutant peptides of N2. Human pol δ activity was assayed as described under Experimental Procedures in the presence of PCNA and varying concentrations (μM) of the mutant peptides of N2. Results are shown as relative activities. Pol δ synthetic peptides used in the assay are shown below the figure (mutated amino acids are marked in boldface letters).

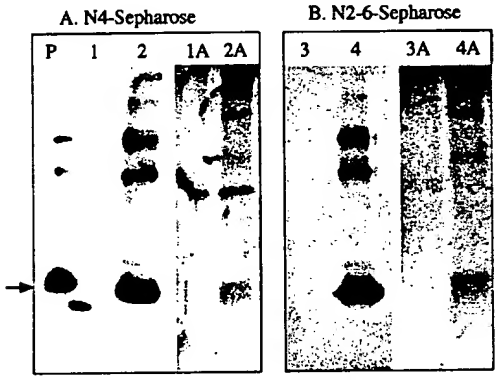


FIGURE 5: Binding of PCNA to immobilized N4 and N2−6 peptides. N4 and N2−6 peptides were covalently coupled to CH-Sepharose. The Sepharose beads (40 μL) were then used to bind purified recombinant human PCNA (40 μL, 0.1 μg/μL). After stringent washing to remove nonspecifically bound material, the beads were extracted with SDS-buffer and subjected to SDS−PAGE and Western blotting using an antibody to PCNA (see Experimental Procedures). Panel A: binding of PCNA to N4-Sepharose. Lane P, purified PCNA; lane 1, binding of PCNA to BSA−Sepharose; lane 2, binding of PCNA to N4-Sepharose; lanes 1A and 2B, silver stain of eluates corresponding to lanes 1 and 2. Panel B: binding to N2−6-Sepharose. Lane 3, binding of PCNA to BSA−Sepharose; lane 4, binding of PCNA to N2−6-Sepharose; lanes 3A and 4B, silver stain of eluates corresponding to lanes 3 and 4. The arrow marks the position of PCNA.

motif (KA-box) that is able to bind PCNA. This was confirmed by a positive overlay of the flagellin−thioredoxin fusion proteins with biotinylated PCNA. As a result of database searches, it was noted that the N4 region of pol δ contains a sequence that conforms to the KA-box. Experimental tests by the use of synthetic peptides in overlay and affinity chromatography experiments showed that the N4 peptide could bind to PCNA. This provides strong evidence that the N4 region of pol δ participates in PCNA binding, and also the first evidence for a functional KA-box in a PCNA-binding protein.

The identification of a KA-box in pol δ is of interest, since we have demonstrated by a number of experimental approaches that the p125 subunit of pol δ interacts with PCNA through a region identified as the conserved N2 region (*37, 39*) which contains a variant of the PIP-box. Thus, the implications of this study are that pol δ interacts with PCNA through the possession of at least two PCNA-binding motifs. A multisite interaction would favor formation of the pol δ−PCNA complex, since it would strengthen the association of the two proteins. Moreover, there is the additional possibility that these interactions could involve different PCNA molecules, since PCNA is a homotrimeric protein. This type of interaction could provide additional strength to the stability of a trimeric PCNA−pol δ complex. Indeed, there is some experimental evidence based on cross-linking experiments which indicates that p125 preferentially forms a complex with trimeric PCNA (*37*).

As already noted under Results, both the PIP-box and the KA-box motifs involve relatively short peptide sequences which do not have very strong signatures, so that database searching provides a large number of hits. This emphasizes the need for experimental verification of the functional ability of any proposed sequence in a given protein to participate in PCNA binding. The identification of a second motif that may be involved in PCNA binding is of particular significance, as it may be of utility in identifying PCNA-binding domains in candidate proteins that bind to PCNA. Furthermore, not all known PCNA-binding proteins contain the PIP-box sequence, and the existence of a second motif may provide an explanation.

The identification of PCNA-binding motifs is of significance since it would provide insights to the structural basis for the versatility of PCNA, given the current information that it is the nexus for multiple protein−protein interactions that underlie its participation in DNA replication, repair, and cell cycle control processes. A role for PCNA in cell cycle control has been indicated by the finding that cyclin/cdks formed quaternary complexes with PCNA and p21 (*19, 40, 41*). The interaction of PCNA with different cyclin/cdks during the cell cycle suggested that the regulated distribution of PCNA could be an important link to cell cycle control of DNA. p21 has been intensively studied since it was discovered to be an inhibitor of the of cyclin/cdks (*42*) and to be transcriptionally regulated by p53 (*43*). p21 inhibits pol δ in vitro and provides an attractive potential molecular mechanism whereby p53 induction could arrest DNA synthesis (*23, 44*) by competing for binding to PCNA (*45, 46*).

Recently, a number of additional proteins that bind to PCNA have been identified (*7−21, 24, 47−52*). These findings have major implications for understanding the role of PCNA. These PCNA-binding proteins fall into three major groups: DNA replication proteins—the catalytic and third subunits of pol δ (*10, 11, 37, 47−49*), RFC (*7−9*), pol ε (*50*), FEN1 (*12, 13*), and DNA ligase I (*14*); DNA repair proteins—XPG (*15*), uracil−DNA glycosylase (UNG2, *16*), mismatch repair proteins MSH2, MLH1, and PMS2 (*51*); cell cycle regulatory proteins—p21 (*22, 23*), p57 (*24*), and the cyclins (*19*). The use of PCNA affinity chromatography has also identified an association of a number of replication and repair proteins with PCNA (*47, 52*). A number of these have been shown to possess functional PIP-boxes, and sequence alignments have provided additional candidates for PCNA binding (*25, 26, 47−49*).

Table 4: Selected Proteins Containing Regions of Similarity to the N4 Regions of Pol δ and the Group III PCNA-Binding Peptides

| | | | | | |
|---|---|---|---|---|---|
| Pol δ (N4 region) | 276 | **RL**K**E**K**A**T**Q**C**Q**L**E**A**D**V**L** | 291 | M01735 |
| Pol ε | 1533 | **K**T**I**C**RAIQ**R**F**L**L**A**Y**K**E** | 1548 | Q07864 |
| RFC-140 | 682 | **K**S**S**L**K**A**I**V**A**E**S**L**N**N**T**S** | 697 | P35251 |
| RFC 38 | 221 | **R**N**L**R**K**A**L**L**M**C**E**A**C**R**V**Q** | 236 | P40938 |
| RFC 37 | 244 | G**D**L**R**K**A**I**T**F**L**Q**S**A**T**R**L** | 259 | P35249 |
| XPC | 712 | **RA**-**RK**A**R**L**A**E**P**Q**L**R**E**E** | 726 | Q01831 |
| XPC (Mouse) | 676 | **RA**-**RK**A**R**H**L**G**A**Q**L**H**D**H** | 690 | P51612 |
| XPD | 224 | E**L**A**R**K**A**V**V**V**F**D**E**A**H**N**I** | 239 | P18074 |
| | 612 | **H**H**Y**G**R**A**V**I**M**F**G**V**P**Y**V**Y** | 627 | P18074 |
| | 630 | S**R**I**L**K**A**R**L**E**Y**L**R**D**Q**F**Q** | 645 | P18074 |
| XPG | 116 | **R**Q**A**I**K**T**A**F**R**S**K**R**D**E**A**L** | 131 | P28715 |
| XPG (xenopus) | 116 | **R**Q**A**I**K**A**A**L**S**G**N**K**Q**S**N**E** | 131 | P14629 |
| UNG2 | 74 | I**Q**R**N**K**A**A**A**L**L**R**L**A**A**R**N** | 89 | X15653 |
| UNG2 (mouse) | 56 | I**Q**R**N**K**A**A**A**L**L**R**L**A**A**R**N** | 71 | P97931 |
| MCM3 | 311 | D**Y**V**K**K**A**I**L**C**L**L**L**G**G**V**E** | 326 | P25205 |
| MCM3 (S. cere) | 374 | D**H**I**K**K**A**I**L**L**M**L**M**G**G**V**E** | 390 | P24279 |
| MCM5 | 706 | **H**A**I**H**K**V**L**Q**L**M**L**R**R**G**E**I** | 721 | P33992 |
| MCM5 (S. cere) | 746 | L**A**L**D**K**A**L**Y**A**L**E**K**H**E**T**I** | 766 | P29496 |
| MCM7 | 348 | E**D**V**K**K**A**L**L**L**L**L**V**G**G**V**D** | 363 | P33993 |
| MCM7 (xenopus) | 347 | E**D**V**K**K**A**L**L**L**L**L**V**G**G**V**D** | 362 | Q91876 |
| MSH6 | 724 | A**I**F**T**K**A**Y**Q**R**M**V**L**D**A**V**T** | 739 | P52701 |
| MSH6 (mouse) | 721 | A**V**F**T**K**A**S**Q**R**M**V**L**D**A**V**T** | 736 | P54276 |
| Cyclin D3 | 92 | V**P**T**R**K**A**Q**L**Q**L**L**G**A**V**C**M** | 107 | P30281 |
| Cyclin D3 (mouse) | 92 | V**P**T**R**K**A**Q**L**Q**L**L**G**T**V**C**L** | 107 | P30282 |
| BRCA 1 | 516 | D**F**I**K**K**A**D**L**A-**V**Q**K**T**P**E** | 530 | P38398 |
| BRCA 1 (mouse) | 509 | D**F**I**K**K**A**D**S**A**G**V**Q**R**T**P**D** | 524 | P48754 |
| BRCA 2 | 2725 | W**Y**A**V**K**A**Q**L**D**P**P**L**L**A**V**L** | 2740 | P51587 |
| BRCA 2 (mouse) | 2646 | W**Y**A**V**K**A**Q**L**D**P**P**L**L**A**V**L** | 2661 | P97929 |

[a] Conserved residues are shown in boldface and are shaded. Accession numbers are shown on the right, and proteins are those of human unless otherwise noted.

As already noted, database searches using the limited sequences of the PIP-box and the KA-box need to be treated with caution. Nevertheless, an intriguing number of proteins that contain the KA-box could be identified. Some of these are shown in Table 4. This list includes members of the MCM (mini chromosome maintenance protein) family, pol ϵ, three XP proteins (XPC, XPG, XPD), mismatch repair protein MSH6, cyclin D3, and BRCA1 and BRCA2. While the possibility that the KA-boxes in these proteins could serve as interaction sites for PCNA binding is purely speculative, evidence that these in fact interact with PCNA and the potential functional significance of such interactions need to be considered.

The nuclear uracil−DNA glycosylase (UNG2) that is involved in base excision repair has been shown to bind both PCNA and RPA (*16*). UNG2 contains a PIP-box at the N-terminus, and two binding sites for RPA (*16*). One of the RPA-binding sites is located between residues 7 and 18, and overlaps the PCNA-binding site. The other site is located between residues 73 and 90 and has the sequence RIQRNKAAALLRLAARNV. Curiously, this site consists of a KA-box (Table 4). Thus, UNG2 contains two sites for RPA, the first of which overlaps the PIP-box, and the second of which conforms to the PCNA-binding KA motif that is described here. Tests by Otterlei et al. (*16*) of the ability of N-terminally truncated UNG2 mutants to bind to PCNA by an ELISA method revealed that the PIP-box was the major determinant for PCNA binding by UNG2; nevertheless, a weak ELISA reaction was observed with a truncated UNG2 mutant in which the PIP-box had been deleted but which still contained the KA-box, leaving open the possibility that this might be due to PCNA interaction with the KA-box.

In summary, we have provided the first evidence for novel peptide motifs for PCNA binding. These findings point to potential relationships that may be important to explore, and also provide a starting point for site-directed mutational studies that could provide evidence for the identities of the PCNA-binding regions. Clearly, much further work needs to be done to establish the function of these motifs in PCNA binding.

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# Caloxin: a novel plasma membrane $Ca^{2+}$ pump inhibitor

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**Chaudhary, Jyoti, Mandeep Walia, Jaswinder Matharu, Emanuel Escher, and Ashok K. Grover.** Caloxin: a novel plasma membrane $Ca^{2+}$ pump inhibitor. *Am J Physiol Cell Physiol* 280: C1027–C1030, 2001.—Plasma membrane (PM) $Ca^{2+}$ pump is a $Ca^{2+}$-$Mg^{2+}$-ATPase that expels $Ca^{2+}$ from cells to help them maintain low concentrations of cytosolic $Ca^{2+}$. There are no known extracellularly acting PM $Ca^{2+}$ pump inhibitors, as digoxin and ouabain are for $Na^+$ pump. In analogy with digoxin, we define caloxins as extracellular PM $Ca^{2+}$ pump inhibitors and describe caloxin 2A1. Caloxin 2A1 is a peptide obtained by screening a random peptide phage display library for binding to the second extracellular domain (residues 401–413) sequence of PM $Ca^{2+}$ pump isoform 1b. Caloxin 2A1 inhibits $Ca^{2+}$-$Mg^{2+}$-ATPase in human erythrocyte leaky ghosts, but it does not affect basal $Mg^{2+}$-ATPase or $Na^+$-$K^+$-ATPase in the ghosts or $Ca^{2+}$-$Mg^{2+}$-ATPase in the skeletal muscle sarcoplasmic reticulum. Caloxin 2A1 also inhibits $Ca^{2+}$-dependent formation of the 140-kDa acid-stable acylphosphate, which is a partial reaction of this enzyme. Consistent with inhibition of the PM $Ca^{2+}$ pump in vascular endothelium, caloxin 2A1 produces an endothelium-dependent relaxation that is reversed by $N^G$-nitro-L-arginine methyl ester. Thus caloxin 2A1 is a novel PM $Ca^{2+}$ pump inhibitor selected for binding to an extracellular domain.

adenosine 5′-triphosphatase; plasma membrane calcium ATPase; transport; calcium; endothelium; erythrocytes; homeostasis; signal transduction

PLASMA MEMBRANE CALCIUM PUMPS are $Ca^{2+}$-$Mg^{2+}$-ATPases that use the energy of hydrolysis of ATP to expel cellular $Ca^{2+}$. Plasma membrane (PM) $Ca^{2+}$ pumps are present in all mammalian cells. However, either PM $Ca^{2+}$ pumps or $Na^+/Ca^{2+}$ exchanger (8, 9, 17) may remove $Ca^{2+}$ from the cell after activation or during homeostasis, and the precise role of PM $Ca^{2+}$ pumps remains to be determined. There are no extracellular PM $Ca^{2+}$ pump inhibitors available to resolve this issue. In contrast, the $Na^+$ pump inhibitors ouabain and digoxin have been pivotal in our understanding of how this pump affects cell function (7, 12). The sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase (SERCA) inhibitor thapsigargin, which can diffuse into the cell to act, has also proved very useful in elucidating the role of the SERCA pump (15). PM $Ca^{2+}$ pumps are encoded by four plasma membrane $Ca^{2+}$-ATPase (PMCA) genes, and their transcripts can be alternatively spliced (2, 4, 10, 14). The isoform PMCA1b is most widely expressed. The PM $Ca^{2+}$ pump protein models show ten transmembrane and five extracellular domains (4). Except for the first putative extracellular domain, their sequences are conserved in different isoforms. All the known functions of the pump are assigned to the cytosolic domains, although mutagenesis of key residues in the PM $Ca^{2+}$ pump protein shows that transmembrane domains are involved in its activity (5). X-ray diffraction studies of the sarcoplasmic reticulum $Ca^{2+}$ pump also support a role for transmembrane domains in the pump activity (16). In contrast, it is unknown whether extracellular domains in the PM $Ca^{2+}$ pump play any role in its function. Earlier, we raised antibodies against solubilized and purified whole PM $Ca^{2+}$ pump protein (1). However, this approach gives antibodies predominantly against cytosolic domains because these form the bulk of the protein and contain many antigenic epitopes. We have now screened a random peptide phage display library to select for peptides binding to the second extracellular domain sequence of the PM $Ca^{2+}$ pump. This domain links transmembrane domains 3 and 4, and mutagenesis of key residues in domain 4 has been shown to be inhibitory (5). The selected peptide inhibits the pump activity. To our knowledge, this is the first extracellular inhibitor of the PM $Ca^{2+}$ pump.

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## METHODS

*Screening of phage display library.* The second putative extracellular domain of PMCA1b in rabbit contains the residues 401–413 (KRPWLAECTPIYI; GenBank accession no. X59069). Including additional amino acids flanking this sequence, replacing serine for cysteine, and adding a cysteine at the COOH terminus, we synthesized the peptide PMCA398 (WVQKRPWLAESTPIYIQYFVKC). PMCA398 was conjugated to keyhole limpet hemocyanin (KHLH) or ovalbumin. A random 12-amino acid mitogen-activated protein or extracellularly regulated kinase 13 phage display library (PhD12; New England Biolabs) was panned for a phage that bound PMCA398-KHLH conjugate dissolved in phosphate-buffered saline (PBS) and eluted with the PMCA398-ovalbumin conjugate. PBS contained 137 mM NaCl, 2.7 mM KCl, 8 mM $Na_2HPO_4$, and 1.5 mM $KH_2PO_4$ (pH 7.4). After eight rounds of panning, elution was performed with PBS for 1, 2, 4, 8, 16, and then 120 min, each time replacing the old PBS. DNA from 20 clones of 16- and 120-min eluants was sequenced. Many clones had no inserts, but six clones encoded for the peptide VSNSNWPSFPSS. Because in the phage the sequence GGG follows the random peptide, the peptide VSNSNWPSFPSSGGG-amide was synthesized and eventually termed caloxin 2A1. Randomization of residues in caloxin 2A1 peptide gave the sequence SWSSFPGSGGVSNPN-amide, and this peptide was synthesized for use as a control.

*Biochemical assays.* Human erythrocyte leaky ghosts were isolated as previously described (6). The ghosts were suspended in 40 mM imidazole-HCl (pH 7.0) and stored at −80°C until use. The skeletal muscle sarcoplasmic reticulum membrane vesicle preparation was a gift from Dr. N. Narayanan of the University of Western Ontario. The $Ca^{2+}$-$Mg^{2+}$-ATPase assays were performed at 37°C by following the disappearance of NADH with a fluorometer (excitation at 340 nm and emission at 460 nm) at 37°C in a coupled enzyme assay that was previously described (1, 9). Basal $Mg^{2+}$-ATPase was first determined in a 135-μl solution that contained 0.2–0.4 mg ghost protein, 0.1 mM ouabain, 100 mM NaCl, 20 mM KCl, 6 mM $MgCl_2$, 30 mM imidazole-HCl (pH 7.0), 0.5 mM EDTA, 0.6 mM NADH, 1 mM phospho*enol*pyruvate, excess pyruvate kinase-acetate dehydrogenase, 0.5 mM ATP, 0.5 mM EGTA, and 4 μg/ml calmodulin. Ten microliters of 8 mM $CaCl_2$ was then added, and the total ATPase activity was determined. The difference between the total ATPase and the basal $Mg^{2+}$-ATPase was the $Ca^{2+}$-$Mg^{2+}$-ATPase activity. $Na^+$-$K^+$-ATPase was assayed in the same solution that was used for the basal $Mg^{2+}$-ATPase except that ouabain was omitted. The $Ca^{2+}$-dependent formation of 140-kDa acid-stable acylphosphates was determined with SDS-polyacrylamide gels at pH 4.0 as previously described (3, 18). The acylphosphates were quantified with a PhosphorImager by following the radioactivity in each band.

*Contractility experiments.* Rats were euthanized with 0.5 ml methoxyflurane, and the thoracic aorta was removed. Aortic rings (3 mm long) with endothelium intact were hung in organ baths under 1.5 g of tension in Krebs solution that contained (in mM) 115 NaCl, 5 KCl, 22 $NaHCO_3$, 1.7 $CaCl_2$, 1.1 $MgCl_2$, 1.1 $KH_2PO_4$, 0.3 EDTA, and 7.7 glucose. Contractions with phenylephrine (0.5 μM) were monitored as previously described (13). Relaxation of the arteries was first monitored using 1 μM carbachol. The arteries were washed for 60 min and then treated again with phenylephrine. After the arteries had reached a steady contraction, caloxin 2A1 dissolved in Krebs solution was added. After another 20–30 min, 100 μM $N^G$-nitro-L-arginine methyl ester (L-NAME) was added (11).

*Data analysis.* Each experiment was replicated at least four times. Values given are means ± SE. Where applicable, Student's *t*-test was used, and values of $P < 0.05$ were considered to be statistically significant. Nonlinear regression was used to determine inhibition constant with the software FigP.

## RESULTS

*Effect of caloxin 2A1 on $Ca^{2+}$-$Mg^{2+}$-ATPase.* Figure 1*A* shows the effect of different concentrations of caloxin 2A1 on $Ca^{2+}$-$Mg^{2+}$-ATPase in erythrocyte leaky ghosts. Caloxin 2A1 inhibits the $Ca^{2+}$-$Mg^{2+}$-ATPase. It produces 50% inhibition at 0.4 ± 0.1 mM. Figure 1*B* shows the selectivity of the inhibition. At a concentration of 0.9 mM, caloxin 2A1 inhibits the $Ca^{2+}$-$Mg^{2+}$-ATPase in the erythrocyte ghosts by 78 ± 4%, but it has no effect on $Mg^{2+}$-ATPase or $Na^+$-$K^+$-ATPase in the ghosts or $Ca^{2+}$-$Mg^{2+}$-ATPase in the skeletal muscle sarcoplasmic reticulum (Fig. 1*B*). Thus caloxin 2A1 inhibits $Ca^{2+}$-$Mg^{2+}$-ATPase selectively. Figure 1*B* also shows that the sequence of caloxin 2A1 is needed to cause the inhibition and not just its amino acid composition, because a randomized peptide with the same amino acid composition does not produce an inhibition.

*Effect of caloxin 2A1 on $Ca^{2+}$-dependent acylphosphate formation.* The second assay of PM $Ca^{2+}$-$Mg^{2+}$-ATPase is based on the $Ca^{2+}$-dependent formation of the acid-

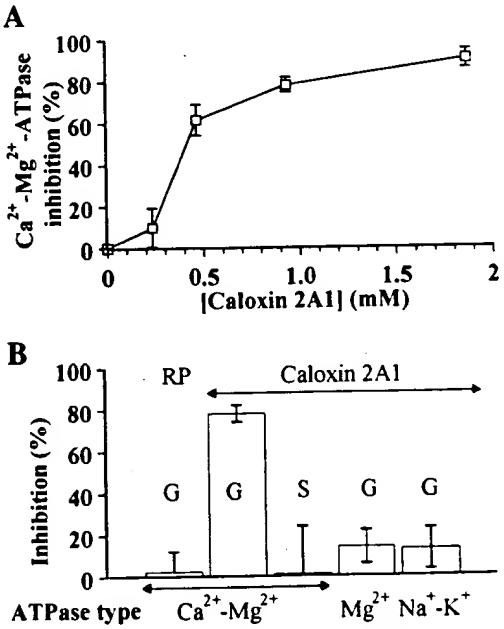


Fig. 1. Effect of caloxin 2A1 on $Ca^{2+}$-$Mg^{2+}$-ATPase. *A*: caloxin 2A1 concentration dependence. The graph shown is from a total of 32 data points pooled from experiments on 6 different days using different preparations of ghosts. On each day, the data were normalized, with the mean value of the activity in the absence of caloxin 2A1 taken as 100%. SE values are shown as error bars. *B*: selectivity of caloxin 2A1 for inhibition of the plasma membrane $Ca^{2+}$-$Mg^{2+}$-ATPase using 0.9 mM caloxin 2A1 or random peptide (RP). G, ghosts; S, skeletal muscle sarcoplasmic reticulum. The types of activity monitored are shown as $Ca^{2+}$-$Mg^{2+}$-ATPase, $Mg^{2+}$-ATPase, or $Na^+$-$K^+$-ATPase. The only significant ($P < 0.05$) inhibition was with caloxin 2A1 in the ghosts.

stable, alkali labile 140-kDa acylphosphate intermediate from [$\gamma$-$^{32}$P]ATP in the erythrocyte leaky ghosts. Because 0.4 ± 0.1 mM caloxin 2A1 produces a 50% inhibition of PM $Ca^{2+}$-$Mg^{2+}$-ATPase, it is expected to cause ~90% inhibition at 3.4 mM. At this concentration, caloxin 2A1 also produces nearly complete inhibition of the acylphosphate formation, but the same concentration of the randomized peptide has no effect (Fig. 2).

*Effect of caloxin 2A1 on endothelium-dependent relaxation.* Inhibition of the PM $Ca^{2+}$ pump in vascular endothelium would increase the concentration of cytosolic $Ca^{2+}$, thereby activating the endothelial nitric oxide synthase, which is $Ca^{2+}$ and calmodulin dependent. The increased nitric oxide relaxes arteries; L-NAME inhibits the nitric oxide synthase (11). In rat aortic rings precontracted with a submaximum concentration (0.5 μM) of phenylephrine, caloxin 2A1 produces a relaxation that is reversed by L-NAME (Fig. 3). Thus caloxin 2A1 produced the biological effect in the endothelium, as expected, from its inhibition of the PM $Ca^{2+}$ pump, although alternative interpretations cannot be completely ruled out.

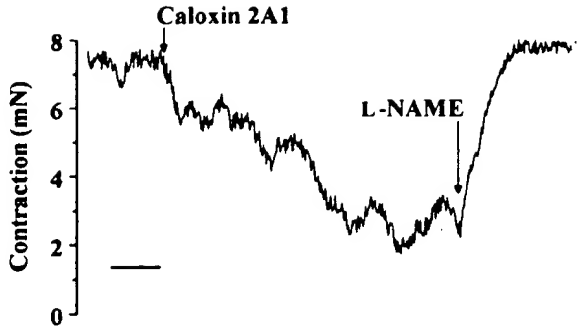


Fig. 3. Endothelium-dependent relaxation of rat aorta. Rat aortic rings (3–4 mm long) were hung in organ baths with the endothelium intact. The rings were contracted with a submaximum concentration (0.5 μM) of phenylephrine before 0.34 mM caloxin 2A1 and 0.1 mM $N^G$-nitro-L-arginine methyl ester (L-NAME) were added as shown. Horizontal bar, 10 min; mN, millinewtons.

## DISCUSSION

Caloxin 2A1, a peptide selected for binding to the sequence of the putative second extracellular domain of PM $Ca^{2+}$-$Mg^{2+}$-ATPase, inhibits this enzyme in human erythrocyte leaky ghosts and produces an endothelium-dependent relaxation in rat aorta. Here, we focus on sequence identities between PMCA isoforms, the relationship of this work to literature on the mechanism of action of the PM $Ca^{2+}$ pump, and the potential applications of caloxin 2A1 to the study of cell function.

The sequence of extracellular domain 401–413 in human PMCA1b (KRPWLAECTPIYI) is similar to the corresponding sequences in PMCAs 2, 3, and 4 (Swiss Protein Bank accession nos. P20020, Q01814, Q16720, and P23634), except that in PMCAs 2 and 4, the residue A is replaced by P, and in PMCA3, the sequence KRP is replaced by GRT. Caloxin 2A1 was obtained by selecting a phage that would bind the PMCA1b sequence; however, it produces a complete inhibition of PM $Ca^{2+}$-$Mg^{2+}$-ATPase in erythrocyte ghosts that expresses mainly PMCA4 (4, 14). This suggests that caloxin 2A1 would inhibit all the PMCA isoforms, but it remains to be established. Furthermore, one cannot rule out different affinities of caloxin 2A1 for individual isoforms. However, caloxin 2A1 was selective for PM $Ca^{2+}$-$Mg^{2+}$-ATPase in that it had no effect on basal $Mg^{2+}$-ATPase, $Na^+$-$K^+$-ATPase, or SERCA1 $Ca^{2+}$-$Mg^{2+}$-ATPase. Additional selectivity of caloxin 2A1 is suggested from the analysis that the target sequence used is absent from all the $Na^+/Ca^{2+}$ exchanger protein sequences reported in the Swiss Protein Bank.

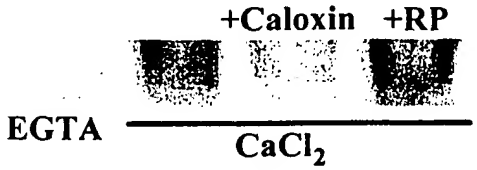


Fig. 2. Acylphosphate formation with 3.4 mM caloxin 2A1 or randomized peptide (RP). The acylphosphate reaction was carried out, and the samples were analyzed by acid gel electrophoresis as previously described. Where shown, EGTA = 1 mM and $CaCl_2$ = 0.05 mM.

Caloxin 2A1 was selected to bind the second putative extracellular domain. Perturbing other domains may also produce inhibition, because mutagenesis of key PMCA residues in putative transmembrane domains 4, 6, and 8 results in a loss of ATPase activity, and the various extracellular domains connect them (5). The sequences of the extracellular domain 1 show the highest diversity between different PMCA isoforms. It is thus possible for this approach to yield peptides that will at least bind more selectively against the extracellular domain 1. This could then lead to isoform-selective caloxins.

This study shows that caloxin 2A1 produces endothelium-dependent relaxation by activating the endothelial nitric oxide synthase, which is a $Ca^{2+}$/calmodulin-activated enzyme (11). The PM $Ca^{2+}$ pump affects virtually every cell, although its role in cell function varies, depending on the expression and level of activity of other transporters. Obviously, this discovery paves the way to examine the role of PM $Ca^{2+}$ pump in tissues with high levels of PMCA expression such as brain, those with high levels of SERCA expression such as skeletal and cardiac muscle, and those with intermediate levels of expression such as platelets, lymphocytes, endothelium, smooth muscle, pancreas, kidney, and liver.

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Observations in this manuscript form the basis of a patent (patent pending).

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# High affinity type I interleukin 1 receptor antagonists discovered by screening recombinant peptide libraries

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ABSTRACT **Two families of peptides that specifically bind the extracellular domain of the human type I interleukin 1 (IL-1) receptor were identified from recombinant peptide display libraries. Peptides from one of these families blocked binding of IL-1α to the type I IL-1 receptor with $IC_{50}$ values of 45–140 μM. Affinity-selective screening of variants of these peptides produced ligands of much higher affinity ($IC_{50} \approx 2$ nM). These peptides block IL-1-driven responses in human and monkey cells; they do not bind the human type II IL-1 receptor or the murine type I IL-1 receptor. This is the first example (that we know of) of a high affinity peptide that binds to a cytokine receptor and acts as a cytokine antagonist.**

The polypeptide cytokine interleukin 1 (IL-1) plays a key role in many immune and inflammatory responses (1, 2). The IL-1 system consists of three distinct but structurally related ligands: IL-1α and IL-1β, which elicit biological responses (3), and IL-1 receptor antagonist (IL-1ra), which acts as an antagonist (4, 5). Two distinct cell-surface receptors have been identified for these ligands, the type I IL-1 receptor (IL-1RtI) (6) and IL-1RtII (7). Most, if not all, of the biological responses to IL-1 result from ligand binding to IL-1RtI (8), and it has been postulated recently that IL-1RtII acts as a decoy receptor (9). In addition to membrane-bound IL-1 receptors, the extracellular portion of IL-1RtII has been reported to be shed (10), and this soluble receptor may act to modulate IL-1 activity.

Elevated IL-1 levels are associated with a number of disease states, particularly autoimmune and inflammatory disorders, and IL-1 antagonists have shown therapeutic value in animal studies and in some clinical settings (11, 12). IL-1ra and soluble IL-1RtI (12, 13) are currently under clinical investigation for treatment of a variety of illnesses. Although these molecules may be effective antagonists, the fact that they are large recombinant proteins may limit their therapeutic application. A small molecule IL-1 antagonist that could be generated synthetically and delivered at high concentrations would be a desirable alternative.

A number of methods have been developed to display biologically generated collections of peptides in a manner which permits facile identification of the displayed peptides through determination of attached DNA sequences. These collections provide enormous amounts of molecular diversity and have been used to identify compounds that bind to biological targets, such as cell-surface receptors or antibody molecules (for a review, see ref. 14). We have generated libraries of random 8-, 10-, 11-, and 12-mer peptides expressed on the N terminus of fd phage pIII protein or the N terminus of the fd phage major coat protein pVIII (14). Here we describe the use of these recombinant peptide display libraries to identify small peptides that bind with high affinity to the human type I IL-1R, block the binding of both IL-1α and IL-1β, and specifically inhibit IL-1-mediated cellular responses.

## MATERIALS AND METHODS

**Strains, Cell Lines, Soluble Receptor, Monoclonal Antibodies, and IL-1R–Fc Fusion.** Phage expressing peptides on the N terminus of pIII were propagated in *Escherichia coli* K91 (15). Phagemid libraries expressing peptides on the N terminus of either pIII or pVIII were generated in *E. coli* ARI 236 [F′(pCJ105) *thr ΔlacX74 galU galK hsdR17 mcrB rpsL(strA) thi*] and propagated during panning in the recA strain derived from K91, ARI 292 (*Hfr-Cavalli thi recA::cat*). The full-length and extracellular domains (ECD) of IL-1RtI were cloned by PCR from HepG2 cell total RNA. The full-length receptor fragment was PCR-amplified with a 5′ *Not*I-containing primer (5′-ctcgcggccgccATGAAAGTGTTACTCAGACTT-3′) and a 3′ *Eco*RI site containing primer 5′-atcgaattCTACCCGAGAGGCACGTGAGCCT-3′. The resulting DNA was then digested with *Not*I and *Eco*RI and cloned into those sites in pSRAlphaNeo (16). The phosphatidylinositol glycan (PIG)-tailed ECD was cloned using the 5′ *Not*I primer (above) and a 3′ primer that adds the Kemptide sequences (17) and a *Not*I site (5′-caggcggccgcacctaagctagctctccggagctcCTTCTGGAAATTAGTGACTGGAT-3′). This ECD PCR fragment was digested with *Not*I and cloned into the *Not*I site of pSRAlpha+HPAP20. In the primers above, the residues designated with capital letters represent homology to the IL-1RtI sequence reported by Chua and Gubler (18). Stable G418-resistant Chinese hamster ovary (CHO) cell lines, soluble receptor (referred to as PIG-R harvest), purified IL-1RtI ECD, and nonblocking monoclonal antibodies were generated as described (17). Ab179 recognizes the 20-aa human placental alkaline phosphatase-derived tail (17), whereas Ab79 binds elsewhere to the IL-1RtI ECD. The extracellular portion of IL-1RtII was cloned by PCR from Raji cell (19) total RNA and inserted into pSRalpha+HPAP20 in a manner analogous to that used for the IL-1RtI ECD. Stable CHO cell lines expressing IL-1RtII were created, and soluble receptor was generated as described for IL-1RtI above.

The bivalent IL-1R–Fc fusion was constructed in a manner similar to other immunoglobulin fusion proteins (20). The

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Abbreviations: IL-1, interleukin 1; IL-1R, interleukin 1 receptor; IL-1ra interleukin 1 receptor antagonist; IL-1RtI, type I IL-1 receptor; IL-1RtII, type II IL-1 receptor; ECD, extracellular domain; CHO, Chinese hamster ovary; EGF, epidermal growth factor; DMEM/F12, DMEM/nutrient mixture F12.

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constant regions of the heavy chain subsegments 2 and 3 of the human Ig Cg1 (gamma1) (21) were cloned from human spleen cDNA (CLONTECH) with primers that add a *Hin*dIII site to the "amino" side and a kemptide sequence, stop codon, and *Xba*I site and *Bam*HI site to the "carboxyl" side. The PCR product was digested with *Hin*dIII and *Bam*HI and cloned into SRa+ digested with *Hin*dIII and *Bam*HI. The IL-1RtI ECD was then cloned into this vector with *Not*I and *Hin*dIII. Stable CHO cell lines were created, individual clones were examined for Ig fusion production by ELISA, and fusion proteins were purified on a protein A column.

**Construction and Screening of Phage Libraries.** Libraries of random peptides expressed at the N terminus of fd phage pIII protein (22) or on the N terminus of the fd phage major coat protein pVIII were created as described (22, 23). In addition to these unbiased libraries, mutagenesis libraries were constructed as described (14, 23).

For panning and ELISAs, the ECD of IL-1RtI was immobilized in wells of an Immulon-4 microtiter plate (Dynatech) in the following manner. Wells were coated with 3 $\mu$g of Ab79 in PBS overnight at 4°C, washed with PBS, and blocked for 1 hr at room temperature with PBS containing 1% BSA. Fifty microliters of PIG-R harvest plus 50 $\mu$l of binding buffer [RPMI 1640 medium with glutamine (Mediatech, Washington, DC) containing 1% BSA, 20 mM Hepes, and 0.1% sodium azide] were added, and plates were placed at 4°C for 2 hr.

Phage libraries were screened as described (22, 23) against immobilized IL-1RtI ECD. After three or four cycles of enrichment, individual phage clones were isolated and analyzed in an ELISA format (24). Binding of phage to IL-1RtI ECD was compared with binding to wells coated with Ab79 alone. DNA was prepared and sequenced as described (22) for receptor-specific phage to deduce the sequence of the expressed peptide.

**Radioligand Competition Binding Assays.** Assays were performed in 96-well microtiter plates (Immulon-4, Dynatech) coated with IL-1RtI ECD on Ab79 or in 96-well plates (Falcon 3047 plates) containing confluent CHO cells expressing the full-length IL-1RtI. Wells were washed three times with binding buffer, and binding buffer was placed in each well. Peptides were resuspended in dimethyl sulfoxide at 20 mM and diluted in binding buffer to the desired starting concentration. Using a Biomek-1000 robotic system (Beckman), five 3-fold dilutions were prepared, and peptide solution was transferred to duplicate receptor-containing wells. To each well, $^{125}$I-labeled IL-1$\alpha$ (Amersham) was added to a final concentration of 40 pM, and the plates were placed on a shaking platform at 4°C for 2 hr. Plates were washed with PBS using a plate washer (Dynatech), and bound $^{125}$I-labeled IL-1$\alpha$ was eluted for 10 min with 0.1 M NaOH and counted in a $\gamma$ counter (Wallac, Gaithersburg, MD). $IC_{50}$ values were determined by fitting the data to a two-parameter logistic equation with the ALLFIT program (25). Peptides with $IC_{50}$ values of less than 20 nM in the above assay were retested in a 24-well format (Falcon 3847 plates) to minimize the effect of peptide depletion at low peptide concentrations. The 24-well assay differs from the 96 well assay in that lower concentrations of receptor were immobilized and binding was done in 2.5 ml. In all cases, nonspecific binding was determined by the addition of 3 $\mu$M IL-1$\alpha$.

**Colony Lifts.** Phage pools enriched for receptor-specific clones were used to infect *E. coli* ARI 292, plated on inducing media (LB agar containing 0.2% arabinose), and grown overnight at 37°C. Colonies were lifted onto nitrocellulose filters that were placed in blocking buffer (PBS containing 3% nonfat dry milk and 1% BSA) for 1 hr at room temperature with several buffer changes. Filters were rinsed in 0.1× blocking buffer containing 0.05% Tween and probed with $^{33}$P-labeled IL-1RtI ECD (monovalent) or with $^{33}$P-labeled bivalent IL-1R–Fc fusion ($^{33}$P-labeled probe was generated using cAMP-dependent protein kinase A) (17) in 0.1× blocking buffer containing 0.05% Tween for 4 hr at 4°C. Filters were washed three times with 0.1× blocking buffer containing 0.05% Tween and three times with PBS, dried briefly, and exposed to film overnight.

**Epidermal Growth Factor (EGF) Receptor Down-Regulation Assay.** CL160 Rhesus monkey cells (ATCC) were grown to confluence in 24-well microtiter plates (Falcon 3047 plates). Wells were washed with DMEM/nutrient mixture F12 (DMEM/F12) (GIBCO/BRL) containing 0.1% BSA, and dilutions of peptide in DMEM/F12 containing 0.1% BSA were added for 2 min at 37°C. IL-1 was then added at 50 pM for 20 min at 37°C. Plates were placed at 4°C, wells were washed with DMEM/F12 containing 0.1% BSA, and $^{125}$I-labeled EGF (Amersham) was added to 140 pM. After 3 hr at 4°C, wells were washed with DMEM/F12 containing 0.1% BSA, and bound EGF was determined.

**IL-1-Induced Prostaglandin $E_2$ Assay.** Human foreskin fibroblast cells (Clonetics, San Diego) were seeded at $1.5 \times 10^4$ cells per well in a 96-well plate (Falcon 3872 plate) in DMEM/F12 containing 10% fetal bovine serum (FBS) and grown overnight at 37°C in 5% $CO_2$. After washing the wells with DMEM/F12 containing 10% FBS, 150 $\mu$l of media containing IL-1$\alpha$ was added. After incubating for 6 hr at 37°C in 5% $CO_2$, 100 $\mu$l of the cell supernatant was used in the prostaglandin $E_2$ scintillation proximity assay (Amersham) according to the manufacturer's instructions. IL-1$\alpha$ was found to have an $EC_{50}$ of 600 fM in the above assay. To test the effect of peptides, cells were seeded and washed as described above, and 140 $\mu$l of peptide in DMEM/F12 containing 10% FBS was added to each well. After a 30-min incubation at room temperature, 10 $\mu$l of 15 pM IL-1$\alpha$ in DMEM/F12 containing 10% FBS was added. Plates were incubated for 6 hr at 37°C in 5% $CO_2$, and the prostaglandin $E_2$ levels in the supernatants were determined as described above.

**Generation of Synthetic Peptides.** Peptides were prepared by solid-phase synthesis with the aid of a peptide synthesizer (Applied Biosystems; model 431A). F-moc amino acids were purchased from SynPep (Dublin, CA). The peptides were assembled by using the standard protocols of the Applied Biosystems SYNTHASSIST software (version 1.0). In all cases, purification of about 100 mg of crude peptide was by preparative, reverse-phase, HPLC on a $C_{18}$ bonded silicagel column (Millipore, Delta Pak, 50 × 300 mm) using an actonitrile/$H_2O$ gradient (5–75% acetonitrile over 60 min). All peptides were greater than 95% pure (on analytical HPLC) and were characterized by fast atom bombardment mass spectrometry or electrospray mass spectrometry.

## RESULTS

**Screening Peptide Libraries Against Immobilized IL-1RtI ECD.** Four phage libraries, encompassing $1.2 \times 10^{11}$ recombinants, were screened against immobilized IL-1RtI ECD. Peptide sequences for those clones that bound specifically to the IL-1RtI ECD are shown in Table 1. All these IL-1R-binding peptides are related and exhibit the consensus sequence WxxxGLW. The registry of this sequence is highly conserved, with 16 of the 23 sequences containing the WxxxGLW consensus starting at the second residue from the free N terminus. Receptor-specific phage containing the motif WxxxGLW were obtained from all four libraries.

Experiments were performed to determine whether the peptides displayed on these phage clones competed with IL-1 for binding to the receptor. In a phage ELISA, 3 $\mu$M IL-1$\alpha$ did not block the binding of these phage to immobilized IL-1RtI ECD. In addition, synthetic peptides corresponding to clones L12, L9, S14, and R11 did not block binding of radiolabeled IL-1$\alpha$ to the immobilized IL-1RtI ECD at concentrations up to 1 mM. The peptides (at 630 $\mu$M) were also inactive in modifying IL-1 induction of prostaglandin $E_2$ in normal human

Table 1. IL-1RtI-binding peptides identified from combinatorial libraries

| Name | Sequence |
|---|---|
| R1 | W W T D T G L W |
| R11 | W W T D D G L W |
| S4 | W W D T R G L W V W T I |
| D29 | F W G N D G I W L E S G |
| S14 | D W D Q F G L W R G A A |
| N1 | R W D D N G L W V V V L |
| C1 | C W S M H G L W L C |
| T11 | S G M W S H Y G I W M G |
| T12 | G G R W D Q A G L W V A |
| M1 | K L W S E Q G I W M G E |
| F17 | G C W D N T G I W V P C |
| L1 | D W D T R G L W V Y |
| L3 | S L W D E N G A W I |
| L4 | K W D D R G L W M H |
| L6 | Q A W N E R G L W T |
| L7 | Q W D T R G L W V A |
| L8, 11 | W N V H G I W Q E |
| L9 | S W D T R G L W V E |
| L12, 17 | D W D T R G L W V A |
| L13 | S W G R D G L W I E |
| L16 | E W T D N G L W A L |
| L19 | S W D E K G L W S A |
| L20 | S W D S S G L W M D |

Recombinant peptide display libraries containing $10^8$ to $10^{11}$ recombinants were screened against immobilized IL-1RtI ECD. After three or four enrichment cycles, individual clones were analyzed by ELISA (24) on wells containing immobilized IL-1RtI ECD or negative control wells containing antibody alone. For clones that produced a signal only in the receptor-containing wells, the phage DNA was sequenced to infer the sequence of the expressed peptide. Residues indicated in bold were conserved among all of the receptor-specific peptides.

dermal fibroblast cells (Clonetics). Thus, it appears that the WxxxGLW peptides do not inhibit IL-1 binding and, further, that these peptides have no effect on at least one cellular response to IL-1.

To identify a different family of peptides that would block IL-1 binding to IL-1RtI ECD, the library screening protocol was modified to avoid recovery of WxxxGLW phage. A random 12-mer library was constructed that lacked both Trp and Met at positions 2 and 8 by using the NNW motif (where W equals A or T) at these positions in the synthetic oligonucleotide. This library ($1.4 \times 10^8$ recombinants) was screened against immobilized IL-1RtI ECD, and receptor-specific phage were identified. In an ELISA, binding of these phage was blocked by the presence of 3 $\mu$M IL-1$\alpha$. All 12 phage clones sequenced encoded a single peptide sequence, RLVYWQPYSVWQR. The corresponding synthetic peptide, T6 (RLVYWQPYSVQR), inhibited binding of radiolabeled IL-1$\alpha$ to the full-length IL-1RtI expressed in CHO cells with an $IC_{50}$ of 59 $\mu$M (Fig. 1). Although this inhibition of radiolabeled IL-1 binding is consistent with the peptide binding to the same sites involved in cytokine binding, we cannot rule out an allosteric mechanism for the observed inhibition.

A second approach was also used to avoid recovery of phage bearing peptides from the WxxxGLW family. The WxxxGLW site was blocked by including peptide WWTDDGLWASGS-$NH_2$ at 250 $\mu$M while screening an N-terminal random 10-mer library expressed on pVIII against immobilized IL-1RtI ECD. Receptor-specific phage were recovered, and in an ELISA, the binding of these phage was blocked by 3 $\mu$M IL-1$\alpha$. Two receptor-specific peptides were identified. The synthetic peptides CW13 (WEQPYALPLE) and CW11 (REYEQPYALW) blocked binding of IL-1$\alpha$ to the full-length IL-1RtI on CHO cells with $IC_{50}$ values of 45 and 140 $\mu$M, respectively.

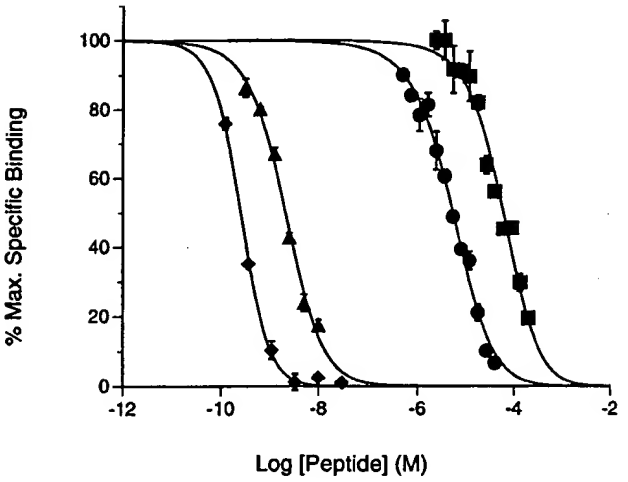


FIG. 1. Inhibition of binding of $^{125}$I-labeled IL-1$\alpha$ to immobilized IL-1RtI ECD by IL-1R-specific peptides. Peptides were identified through use of recombinant peptide display libraries as described in the text. Competition binding was performed as described. In this assay, IL-1ra had an $IC_{50}$ of 1.6 ± 0.9 nM. ◆, IL-1α; ■, peptide T6 (RLVYWQPYSVQR); ●, peptide $^3$H4 (TFVYWQPYALPL); ▲, AF10847 (ETPFTWEESNAYYWQPYALPL).

**Residues Important for Receptor-Specificity.** To identify the specific amino acid residues involved in IL-1R binding as well as to obtain peptides with increased affinity for IL-1RtI, seven mutagenesis libraries (representing $4.3 \times 10^8$ phage clones) were synthesized. Two of these libraries were based on the sequence of the T6 peptide using either a 70–10–10–10 mutagenesis strategy (14) or a codon-by-codon mutagenesis strategy (14). The other libraries kept either the left or right six residues of the T6 (RLVYWQPYSVQR) or 3H4 (TFVYWQPYALPL) peptides fixed and randomized the remaining half using the NNK motif (where K represents an equimolar mixture of G and T). These libraries were panned against immobilized IL-1RtI ECD, and receptor-specific peptides were identified. A total of 161 unique IL-1R-binding peptide sequences were determined; 143 of these were synthesized and tested to determine their $IC_{50}$ values for inhibiting the binding of $^{125}$I-labeled IL-1$\alpha$ to the full-length IL-1RtI on CHO cells. $IC_{50}$ values ranged from 3 to >250 $\mu$M (data not shown). All of the IL-1RtI-binding peptides contain the sequence QPY, with the exception of PG42 (IMWFCQPGGACYSV), which when synthesized as a disulfide cyclized peptide had an $IC_{50}$ of 230 $\mu$M. Immediately N-terminal to QPY are two residues containing primarily aromatic side chains, although some peptides contain alkyl or acidic residues. No particular side chain requirements were noted among residues preceeding these aromatic positions; in fact, peptide CW13 (WEQPYALPLE; $IC_{50}$ = 45 $\mu$M) lacks residues N-terminal to the aromatic positions. Immediately C-terminal to the conserved QPY, an Ala or Ser was found among all the receptor-specific sequences except for PG42 and L15 (SRVWYQPYFVQP; $IC_{50}$ = 41 $\mu$M). The remaining positions do not show any side chain preference. Thus, this peptide family has several conserved residues that appear to contribute to specific binding. However, the method used to identify them did not discriminate among low and high affinity sequences.

**Identification of Analogs with Increased Affinity.** A number of methods can be used to enrich for high affinity ligands on the surface of phage particles. These methods include monovalent peptide display (26, 27) and low density immobilization of target molecules to minimize multivalent interactions (24). To generate high affinity ligands for IL-1RtI, a total of nine peptide libraries that extended portions of the IL-1R binding peptides were designed. These libraries were constructed in the

Table 2. IL-1RtI-binding peptides identified by colony lift

| Name | Sequence | $IC_{50}$, nM |
|---|---|---|
| AF11224 | SVGEDHNFWTS**EYWQPYALPL** | 480 ± 110 |
| AF10817 | ENTYSPNWADS**MYWQPYALPL** | 130 ± 9 |
| AF10847 | ETPFTWEESNAY**YWQPYALPL*** | 2.6 ± 0.3 |
| AF10961 | TANVSSFEWTPG**YWQPYALPL*** | 2.6 ± 0.5 |
| AF11308 | DGYDRWRQSGER**YWQPYALPL*** | 2.1 ± 0.5 |
| AF10816 | SWTDYG**YWQPYALPL**ISGL | 320 ± 25 |

Phage libraries were panned against immobilized IL-1RtI ECD using the affinity enrichment process of ligand-mediated dissociation as described in text. Enriched pools were screened by colony lift as described in *Materials and Methods*. Residues indicated in bold were kept constant, while the remaining residues were encoded using an NNK motif. $IC_{50}$ determinations were performed on synthetic peptides as described, using Ab79 immobilized IL-1RtI ECD. $IC_{50}$ values are an average ± SEM of three independent testings.

*Identified by probing with monovalent, labeled receptor.

pIII phage, pIII phagemid, and pVIII phagemid (23) systems, and comprised a total of $8 \times 10^8$ recombinants. During panning, enrichment of high affinity ligands was accomplished by using ligand-mediated dissociation. After washing away unbound phage, a high concentration of either a competing peptide or a natural ligand (IL-1α in this case) was added to accelerate the dissociation of phage bearing lower affinity ligands (28). This method is effective because the high concentration of competing ligand blocks reassociation of peptides displayed on phage. Phage bearing low affinity peptides dissociate from the receptors, while those expressing high affinity peptides remain bound. The temperature and duration of incubation can be varied to achieve different amounts of dissociation.

To identify phage-expressing peptides with improved affinity for IL-1RtI, the enriched pools were screened by colony lift. For each enriched pool, six plates were screened, containing a total of 12,000–30,000 colonies. Probing of colony lifts with radiolabeled bivalent IL-1R–Fc fusion resulted in a large number of positive phage clones. Those corresponding to the darkest dots (hopefully those with highest affinity for the soluble receptor) were selected for sequencing. The three synthetic peptides derived from these phage sequences had $IC_{50}$ values of 100–500 nM (Table 2). Probing colony lifts with radiolabeled monomeric IL-1RtI ECD resulted in the identification of three clones from over 60,000 screened in the pool of phage that had undergone affinity selection. In a parallel experiment without ligand-mediated dissociation, no colony lift positive clones were observed with monovalent receptor. These three peptides were synthesized, and all were found to inhibit IL-1 binding with an $IC_{50}$ of about 2 nM (Table 2). From the lack of sequence homology among the twelve N-terminal residues of AF10847, AF10961, and AF11308, it is apparent that there are multiple N-terminal extensions that increase the binding affinity of the YWQPYALPL sequence.

A number of N-terminal deletions of these high affinity peptides were synthesized and tested for binding (Table 3). Removal of the three N-terminal residues in AF10847 did not result in a significant loss in affinity. However, removal of Phe-4 resulted in at least a 30-fold drop in affinity. Removal of the six N-terminal residues on AF10961 did not significantly impact affinity. With AF11308, removal of the five N-terminal residues resulted in a 100-fold loss in affinity. The $IC_{50}$ values of the N-terminal truncations reveal that many of the N-terminal residues do not contribute to the affinity of the peptide–receptor interaction. AF10961 appears to have a greater number of expendable N-terminal residues than AF10847 and AF11308. Fig. 1 shows the affinity maturation of the IL-1R-binding compounds from the initial 60 μM lead to the high affinity compounds listed in Table 3. The affinities of the high affinity peptides compare favorably with those obtained for IL-1ra (1.6 ± 0.9 nM).

**Inhibition of IL-1-Driven Cellular Responses.** Binding of IL-1 to cell-surface receptors results in a variety of cellular responses, including stimulation of T-cell proliferation (29) and prostaglandin $E_2$ production (30, 31). Many IL-1-mediated responses are quite rapid and can be easily quantified. These include EGF-R down-regulation (32) and induction of IL-6 (33), IL-8 (34), and adhesion molecules (35). The ability of the peptides described above to inhibit these IL-1-mediated cellular responses was examined. AF11377 blocked IL-1 induction of E-selectin on human umbilical vein endothelial cells (Clonetics) with an $IC_{50}$ of 120 nM (Fig. 2*A*). IL-1ra blocked this response with an $IC_{50}$ of 0.97 ± 0.2 nM). AF10847 blocked the production of IL-8 from human dermal fibroblasts with an $IC_{50}$ of 8.7 nM (Fig. 2*B*). IL-1ra blocked this response with an $IC_{50}$ of 19 ± 6 pM. AF11377 and AF10847 also blocked IL-1-induced IL-6 secretion, prostaglandin $E_2$ accumulation, EGF-R down-regulation in human foreskin fibroblast cells, IL-1-induced intercellular adhesion molecule-1 expression on human umbilical vein endothelial cells, and IL-1-induced IL-6 and IL-8 secretion in cultured whole blood with $IC_{50}$ values in the nM range (data not shown).

**Specificity.** The screening, ELISA, and binding assays described above were performed with a cloned form of the IL-1RtI. The peptides identified here were tested for their ability to block IL-1 binding to a native form of IL-1RtI. Peptide AF10847 blocked binding of IL-1α to normal human dermal fibroblast cells with an $IC_{50}$ of 5.6 nM (data not shown).

Table 3. Truncations of high affinity IL-1RtI-binding peptides

| Name | Sequence | $IC_{50}$, nM |
|---|---|---|
| AF10847 | ETPFTWEESNAYYWQPYALPL | 2.6 ± 0.3 |
| AF12878 | FTWEESNAYYWQPYALPL | 6.3 ± 1.9 |
| AF12877 | TWEESNAYYWQPYALPL | 230 ± 20 |
| AF10961 | TANVSSFEWTPGYWQPYALPL | 2.6 ± 0.5 |
| AF11377 | FEWTPGYWQPYALPL | 1.9 ± 0.1 |
| AF11308 | DGYDRWRQSGERYWQPYALPL | 2.1 ± 0.5 |
| AF11440 | WRQSGERYWQPYALPL | 85 ± 31 |

$IC_{50}$ determinations were performed on synthetic peptides as described, using Ab79 immobilized IL-1RtI ECD. $IC_{50}$ values are an average ± SEM of three independent testings.

IL-1RtI has been reported to bind IL-1$\alpha$ and IL-1$\beta$ with roughly comparable affinity (34). AF10847, AF10961, and AF11308 blocked $^{125}$I-labeled IL-1$\beta$ binding to immobilized IL-1RtI ECD with $IC_{50}$ values of 15, 13, and 11 nM, respectively (data not shown).

The human type I and type II IL-1 receptors share only 28% homology (7). Although IL-1RtI does not discriminate between IL-1$\alpha$ and IL-1$\beta$, IL-1RtII preferentially binds IL-1$\beta$ (10). A soluble form of IL-1RtII was generated in a manner identical to that described for IL-1RtI (see *Materials and Methods*) (17). Binding of $^{125}$I-labeled IL-1$\beta$ to IL-1RtII ECD immobilized on Ab179 was blocked by IL-1$\beta$ and IL-1ra with $IC_{50}$ values of 6.1 and 20 nM, respectively. AF10847, AF10961, and AF11308 did not inhibit binding of $^{125}$I-labeled IL-1$\beta$ to Ab179-immobilized IL-1RtII ECD at the highest concentration tested (90 $\mu$M) (data not shown). These data suggest that the peptides are at least 50,000-fold selective for IL-1RtI.

The human and mouse type I IL-1 receptors share considerable sequence homology, with 64% identity among residues in their ECDs (6). AF10847 did not inhibit binding of human $^{125}$I-labeled IL-1$\alpha$ or human $^{125}$I-labeled IL-1$\beta$ to mouse EL4 cells at a concentration of 12 $\mu$M. AF10847 was also unable to inhibit IL-1-induced IL-6 expression in 3T3 cells at a concentration up to 100 $\mu$M (data not shown). Thus, AF10847 does not appear to bind to the mouse IL-1R. However, these peptides did bind to the monkey IL-1RtI expressed on the Rhesus monkey cell line CL160 (ATCC). Since these cells did not bind detectable levels of human $^{125}$I-labeled IL-1$\alpha$, a functional assay was developed to measure the IL-1 induced reduction of $^{125}$I-labeled EGF binding to EGF-R. AF11377 blocked this IL-1 response with an $IC_{50}$ of 35 nM (data not shown). Thus, AF11377 appears to interact with the Rhesus monkey IL-1R and blocks a physiological response in these cells.

## DISCUSSION

Despite extensive screening efforts by the pharmaceutical industry, it has been difficult to identify high affinity small molecule agonists or antagonists for cytokine receptors. Information from cytokine mutagenesis studies (36–38) and elucidation (by crystallography) of the structure of cytokines bound to their receptors (39, 40) indicate that protein–protein contacts involved in binding and signal transduction are distributed over a large three-dimensional space. On the basis of these findings, some have suggested that the nature of cytokine/receptor interactions might preclude the identification of high affinity small molecule agonists or antagonists. The high affinity peptide ligands reported here demonstrate that this is not the case. The tremendous molecular diversity that was accessed by screening large recombinant peptide libraries has allowed us to identify high affinity antagonists with less than 10% of the mass of the natural cytokine.

It has recently been suggested that despite an extensive protein–protein interface, much of the binding energy for the human growth hormone–human growth hormone binding protein interaction comes from a few contacts (41). In addition, recent mutagenesis studies of IL-1ra suggest that a significant portion of the binding energy of the IL-1ra/IL-1RtI interaction may also be generated in a compact interface of the two proteins (42). The key residues of IL-1ra involved in binding (Trp-16, Gln-20, Tyr-34, Gln-36, and Tyr-147) are discontinuous in the primary sequence but adjacent to one another on the surface of the folded protein. We have recently confirmed the importance of these residues in contacting the IL-1RtI by solving the crystal structure of the IL-1ra/IL-1R complex (H. Schreuder, unpublished data). Our high affinity peptide contains similar S.Y., R.B., A.A. and T.B., residues. Determination of whether the peptide mimics the contact residues of IL-1ra and binds to the same sites on the receptor awaits elucidation of the structure of the peptide/receptor complex.

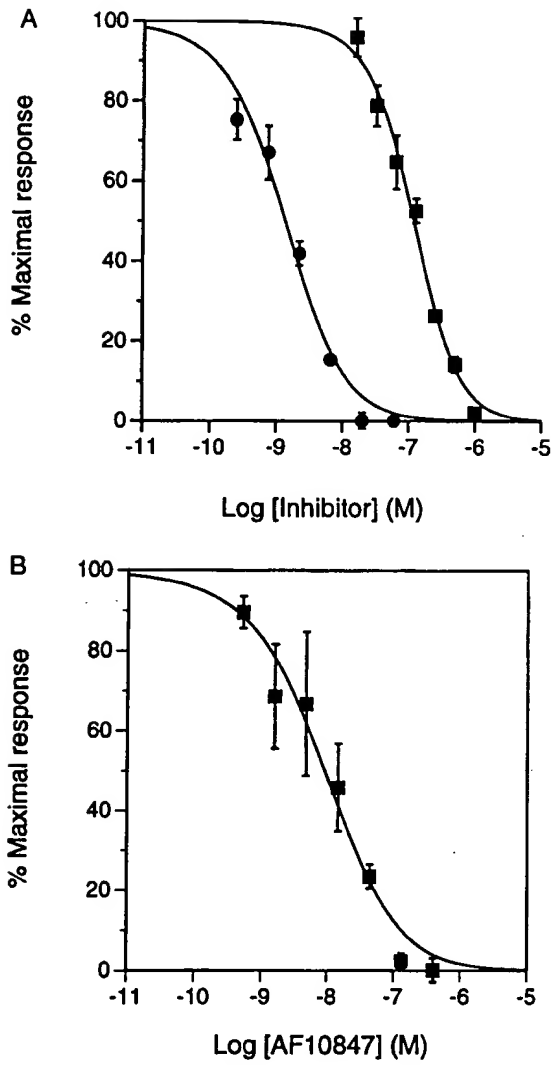


FIG. 2. (*A*) Peptide AF11377 inhibition of IL-1-induced E-selectin in human umbilical vein endothelial cells. Wells in 48-well plates (Costar 3548 plates) containing a confluent lawn of human umbilical vein endothelial cells were prepared and washed with DMEM/F12 containing 0.1% BSA. Dilutions of peptide in DMEM/F12 containing 0.1% BSA and 120 pM IL-1$\alpha$ were added for 20 min at 37°C and removed by washing with DMEM/F12 containing 0.1% BSA, and fresh DMEM/F12 containing 0.1% BSA was added. After 4 hr at 37°C, a monoclonal antibody to E-selectin was added (Ab119.5) at 1:10,000 in DMEM/F12 containing 0.1% BSA. After 30 min at 4°C, wells were washed, and 40,000 cpm/well of $^{125}$I-labeled protein A (Amersham) was added. After 30 min at 4°C, wells were washed with PBS, and bound protein A was eluted with 1 M NaOH and counted in a $\gamma$ counter. ■, AF11377; ●, IL-1ra. (*B*) Peptide AF10847 inhibition of IL-1 induced IL-8 in human fibroblasts. Normal human dermal fibroblast cells were cultured in DMEM with 10% fetal calf serum in 48-well plates. Twenty-four hours before initiation of the experiment, the medium was changed to DMEM with 0.2% fetal calf serum. Peptide antagonist was added to confluent cells, which were then pulsed for 30 min with rIL-1$\beta$ (R & D Systems) at 50 pg/ml. The cells were washed four times with PBS, and fresh DMEM/0.2% fetal calf serum was added to each well. After 5 hr of incubation, the culture media were collected and analyzed by ELISA for IL-8 (R & D Systems). Maximal activation, with IL-1 only, was 230 ± 11 pg/ml of IL-8. Nonspecific activation, determined in the presence of 1000-fold excess IL-1ra, was 32 ± 8 pg/ml of IL-8 ($n$ = 3).

In summary, our results demonstrate the power of methodologies for creating and screening highly diverse recombinant peptide libraries to identify new ligands for a cytokine recpetor. These ligands may serve as important research tools and lead compounds for developing compounds with therapeutic utility.

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# High-affinity urokinase receptor antagonists identified with bacteriophage peptide display

(peptide libraries/cell surface protein/plasminogen activation)

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ABSTRACT **Affinity selection of a 15-mer random peptide library displayed on bacteriophage M13 has been used to identify potent ligands for the human urokinase receptor, a key mediator of tumor cell invasion. A family of receptor binding bacteriophage ligands was obtained by sequentially and alternately selecting the peptide library on COS-7 monkey kidney cells and baculovirus-infected Sf9 insect cells overexpressing the human urokinase receptor. Nineteen peptides encoded by the random DNA regions of the selected bacteriophage were synthesized and tested in a urokinase receptor binding assay, where they competed with the labeled N-terminal fragment of urokinase with $IC_{50}$ values ranging from 10 nM to 10 μM. All of the isolated peptides were linear and showed two relatively short conserved subsequences: LWXXAr (Ar = Y, W, F, or H) and XFXXYLW, neither of which is found in urokinase or its receptor. Competition experiments demonstrated that the most potent peptide, clone 20, prevented binding of bacteriophage displaying the urokinase receptor binding sequence (urokinase residues 13–32). In addition, this peptide blocked other apparently unrelated receptor binding bacteriophage, suggesting overlapping receptor interaction sites for all of these sequences. These results provide a demonstration of bacteriophage display identifying peptide ligands for a receptor expressed on cells and yield leads for the development of urokinase receptor antagonists.**

The migration and invasion of cells are necessary for many normal and pathological processes, including tissue remodeling, embryo implantation, angiogenesis, and tumor cell invasion and metastasis (1–4). Recent reports suggest that these processes require an active cell-surface proteolytic cascade (5, 6). Important components of this cascade are the plasminogen activator/plasmin system, as well as the matrix metalloproteinases (6). The requirement for both protease expression and a cell-surface protease binding protein has been demonstrated most clearly in the case of urokinase plasminogen activator (uPA) and the uPA receptor (uPAR) but has also been recently described for type IV collagenase (7, 8). It has been shown in human colon and breast carcinomas that urokinase is expressed in stromal, fibroblast-like cells and uPAR is expressed on tumor epithelial cells or macrophages, respectively (9, 10). This suggests that a paracrine relationship between uPA and its receptor occurs in these pathological conditions. The *in vitro* observation that human tumor cell invasion is proportional to receptor-bound urokinase, not total urokinase synthesis, further supports the hypothesis that cell-surface protease localization is a key for invasion (11, 12). Other results show that plasminogen activation is more efficient when both uPA and plasminogen are bound on a cell surface, and that cell-surface plasmin is resistant to inhibition by $\alpha_2$-antiplasmin (7). The *in vivo* observation that metastasis of human prostate cancer cells in a nude mouse model is drastically reduced by the expression or administration of a protease-deficient urokinase receptor ligand suggests a role for uPAR antagonists in treating metastatic disease (13).

We have used random peptide bacteriophage display to identify urokinase receptor ligands. Bacteriophage display permits the expression of millions of peptides (14, 15) or proteins (16–18) on the surface of bacteriophage particles, biochemical selection of ligands, and identification by DNA sequencing of the packaged bacteriophage genomes. Peptide ligands for several soluble proteins including streptavidin, concanavalin A, integrins, such as $gpII_bIII_a$, and a variety of antibodies have been identified (15, 19–22) as well as antibody fragments that bind to cells (23). We report here the identification and characterization of peptide antagonists with nanomolar affinity for the human uPAR by using a 15-mer peptide library. This extension of bacteriophage peptide display to cell-surface-expressed proteins expands the utility of the method to a wide variety of biologically interesting targets.

## MATERIALS AND METHODS

**Reagents and Strains.** Bacteriophage library construction and bacteriophage growth and isolation were performed as described by Devlin *et al.* (15). The *Escherichia coli* strains H249, a *recA*, *sup°*, F′ derivative of MM294, and JM103 [F′ *traD36 proAB⁺ lacI^q lacZ*ΔM15 Δ(*pro-lac*) *supE hsdR endA1 sbcB15 thi-1 strA*λ⁻] were used for these experiments. Recombinant DNA manipulations were according to Sambrook *et al.* (24); electrocompetent *E. coli* HB101 (Stratagene) were used for subcloning unless otherwise noted. Restriction enzymes were from New England Biolabs; high molecular weight human uPA, plasminogen, and the anti-uPAR monoclonal antibody 3936 were from American Diagnostica (Greenwich, CT). Streptavidin was from Molecular Probes or Sigma, and bovine serum albumin (BSA) was from Sigma. Immulon-2 96-well plates were from Dynatech. The plasmin substrate S-2251 was from Kabi Pharmacia Diagnostics (Piscataway, NJ). Linear synthetic peptides were prepared on an Applied Biosystems model 430A peptide synthesizer using 9-fluorenylmethoxycarbonyl-based chemistry and were purified by reversed-phase HPLC after trifluoroacetic acid cleavage. Alternatively, peptides were obtained from Chiron Mimotopes (Melbourne, Australia). The cyclic uPA peptide encompassing residues 12–32 with Cys-19 changed to Ala was obtained from V. Huebner (Chiron). Bacteriophage-derived peptides were synthesized with free N termini and C-terminal amides and were characterized by amino acid analysis using the Pico-tag method of Waters. Peptides were typically stored as concentrated stocks in 100% dimethyl sulfoxide at 4°C.

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Abbreviations: uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; ATF, N-terminal fragment of urokinase; EGF, epidermal growth factor; BSA, bovine serum albumin.

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**Cloning and Expression of Cell-Surface uPAR.** A full-length human uPAR cDNA was isolated by PCR from a bacteriophage λ lysate DNA preparation of a phorbol 12-myristate 13-acetate-stimulated U937 cell cDNA library obtained from P. Olson (Chiron), using the following oligonucleotide primers, based on the published sequence (25): N terminus, 5′-CTAGAAGCTTATGGGTCACCCGCCGCTGCTG-3′; and C terminus, 5′-CGTAGTCGACTTAGGTCCAGAGGAGAGTGC-3′. PCRs were performed in 100 μl with the following components: 10 mM Tris·HCl, pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.2 mM each dATP, dGTP, dTTP, dCTP/1 μM sense and antisense primer/100 ng of template cDNA preparation/2.5 units of *Taq* DNA polymerase (Perkin–Elmer/Cetus). The reaction conditions were 94°C (1 min), 37°C (2 min), and then 72°C (3 min) for 30 cycles. The resulting PCR fragment was subcloned into *Hin*dIII/*Sal* I-digested pBR322, and its identity with the published sequence was confirmed by DNA sequencing. To transfer the full-length sequence to pAc373 (a baculovirus expression vector), the subclone was digested with *Hin*dIII/*Sal* I; the ends of the isolated fragment and the *Bam*HI linearized expression vector were filled in with Klenow fragment and deoxynucleoside triphosphates and then ligated to yield pflu-PAR, the expression vector.

Cell-surface human uPAR expression in COS-7 cells was accomplished by using the Mo3 cDNA in the vector pCDNA1 as described (26).

**Expression and Purification of Soluble Recombinant Proteins.** A truncated, soluble version of human uPAR was secreted from baculovirus-infected Sf9 insect cells. The expression vector encoding the soluble receptor (psu-PAR) was obtained by digesting the pBR322 subclone of the full-length receptor with *Sph* I/*Drd* I, yielding a fragment encoding amino acids 27–278 of uPAR. The sets of adaptors shown below were ligated to the 5′ end, introducing a *Bgl* II site and restoring the original first 26 amino acids, and to the 3′ end replacing amino acids Val and Glu (positions 278 and 279) with Leu and Val, while introducing a premature stop codon at position 280. For the 5′ end:

```
5'-GATCTATGGGTCACCCGCCGCTGCTGCCGCTGCTGCTGCT -
    3'-ATACCCAGTGGGCGGCGACGACGGCGACGACGACGA -

GCTCCACACGTGCGTCCCAGCCTCTTGGGGCCTGCGGGTGCATG-3'
CGAGGTGTGCACGCAGGGTCGGAGAACCCCGGACGCCAC-5'
```

For the 3′ end:

```
5'-ATCTTGTTTAAA-3'
3'-CCTAGAACAAATTTCTAG-5'
```

The resulting fragment was cloned directly into the *Bam*HI site of pAc373, and correct clones were validated by restriction analysis and DNA sequencing. Soluble uPAR was purified from conditioned medium of baculovirus-infected Sf9 cells, by DEAE ion-exchange and Sephacryl S-100 size-exclusion chromatography, followed by preparative C-4 reversed-phase HPLC (S.R. and R. Yamamoto, unpublished data).

**Receptor Binding Assay.** Purified soluble uPAR was biotinylated with NHS-biotin (Molecular Probes) and immobilized at 0.3 μg/ml in phosphate-buffered saline (PBS)/0.1% BSA on streptavidin-coated Immulon-2 96-well Removawell plates (27). Human uPA N-terminal fragment (ATF; from M. Shuman, University of California, San Francisco) was iodinated by the Iodo-Gen method (Pierce). Unincorporated $^{125}I$ was separated from labeled protein by Sephadex G-25 chromatography. The specific activity of the labeled protein was between $5 \times 10^5$ and $1 \times 10^6$ dpm/pmol. The iodinated tracer (100–500 pM) was incubated with synthetic peptides in triplicate for 2 h at room temperature in PBS/0.1% BSA in a total vol of 200 μl. The plates were washed three times with PBS/0.1% BSA, and remaining bound radioactivity was measured on an LKB 1277 Gammamaster. Scatchard analysis was performed by using the LIGAND program (Biosoft, Milltown, NJ) (28).

**Construction of a Positive Control Bacteriophage.** A positive control bacteriophage, encoding human uPA residues 13–32 with Cys-19 converted to Ala (13-32C19A), was constructed by PCR with the M13 bacteriophage derivative pLP67 (15) as template. The sense primer for the reaction was 5′-TTTAGTGGTACCTTTCTATTCTCACTCCGCTGAATGTCTAAATGGAGGAACAGCTGTGTCCAACAAGTACTTCTCCAACATTCACTGGTGCAACCCGCCTCCACCTCCACCGACTGTTGAAAGTTGTTTAGC-3′, encoding an in-frame N-terminal *Kpn* I site in the P3 gene at position 1615 of pLP67. This *Kpn* I site is followed by a P3 sequence through the first 2 residues of the mature P3 protein (Ala-Glu), residues 13–32 of the epidermal growth factor (EGF)-like domain of human uPA with Cys-19 changed to Ala, and 6 proline residues separating the uPA sequence from P3. The antisense primer 5′-AAAGCGCAGTCTCTGAATTTACCG-3′ encodes the *Alw*nI site at position 2192 of pLP67. The PCR (38 cycles) was carried out as described above and the products were ethanol precipitated, resuspended, and digested with *Kpn* I and *Alw*nI. The major product of ≈640 bp was isolated from a 1% low melting point agarose gel by agarase digestion and was ligated with the purified large *Kpn* I/*Alw*nI-digested fragment of pLP67. After transformation, insert positive clones were identified by PCR and were confirmed by DNA sequencing.

**Peptide Library Affinity Selection.** Sf9 insect cells ($10^6$), infected 48 h previously with a baculovirus expressing the human substance P receptor, were incubated with $10^{10}$ library bacteriophage for 30 min at room temperature in 0.5 ml of Grace's insect cell medium containing 2% nonfat dry milk (GBB) to remove nonspecifically adherent bacteriophage. Cells were removed by centrifugation and the bacteriophage-containing supernatant was incubated for 30 min with gentle agitation with $10^6$ Sf9 cells displaying human uPAR. Cells were then washed five times with 10 ml of GBB, and bacteriophage were eluted with 0.5 ml of 6 M urea (pH 3) for 15 min at room temperature. The eluate was neutralized with 2 M Tris base (10 μl), and the bacteriophage yield was determined by titering on *E. coli* JM103. Bacteriophage were then amplified as plaques on large agar plates at $\approx 2 \times 10^5$ plaques per plate as described (15). Bacteriophage were eluted with Tris-buffered saline for 6 h, precipitated with polyethylene glycol 8000 (Sigma), and stored at 4°C at $\approx 10^{13}$ plaque-forming units/ml. Eluted, amplified bacteriophage were then affinity selected on transfected COS-7 cells overexpressing human uPAR. Cells, transfected 48 h previously by the DEAE-dextran method with the uPAR expression plasmid (26), were washed once with Dulbecco's modified Eagle's high glucose medium containing 2% nonfat dry milk and 20 mM Hepes (pH 7.2) (DBB). Cells were incubated with $\approx 10^{11}$ bacteriophage from the round 1 eluate for 30 min at room temperature and washed 10 times with 5 ml of DBB; bound bacteriophage were eluted as described above. Eluted bacteriophage were amplified and $10^{10}$ infectious particles were used as input for a third round of affinity selection on uPAR-expressing Sf9 cells, as described for round 1. Bacteriophage eluted from this final round of selection were plated, and individual plaques were picked and analyzed by DNA sequencing.

**Peptide Bacteriophage Competition Experiments.** *E. coli* JM103 were infected with bacteriophage and grown for 6–8 h, and bacteriophage particles were isolated by centrifugation and polyethylene glycol precipitation. Immulon-2 96-well plates were coated overnight at 4°C with 10 μg of streptavidin per well in 100 μl of 0.1 M sodium bicarbonate (pH 9.0). The wells were drained, washed once with 300 μl of PBS, and

blocked with 200 μl of 5% BSA in PBS for 1 h at room temperature with gentle agitation. The wells were rinsed three times with PBS and 1 μg of biotinylated uPAR was added and incubated for 1 h, drained, and washed three times with 300 μl of PBS. Aliquots of fresh bacteriophage stocks were mixed with either PBS or 2 μM peptide in 0.2% dimethyl sulfoxide. Two peptides were used: clone 20, AEPMPHSLNFSQYLWYT; control peptide, AEWVWPTEDSPTPSYDY (WVWP). These samples (100 μl) were incubated in uPAR-coated wells for 2 h at room temperature, the wells were washed 10 times with 300 μl of PBS, and remaining bacteriophage were eluted with 100 μl of 0.1 M glycine (pH 2.2) for 15 min at room temperature. Eluted bacteriophage were neutralized with 20 μl of 1.5 M Tris·HCl (pH 8.8) and titered in duplicate for the inputs and in triplicate for the eluates as described (15).

## RESULTS

To affinity select the bacteriophage peptide library, we first cloned and expressed human uPAR, a glycosyl phosphatidylinositol-linked integral membrane protein of 313 amino acids (25, 29). A full-length receptor cDNA was inserted into vectors for expression in mammalian cells (26) and for production of a recombinant baculovirus. Transfection of the plasmid into COS-7 cells or infection of Sf9 cells with the recombinant baculovirus yielded cells that displayed high levels of functional uPAR, as shown by immunological detection with an anti-receptor monoclonal antibody and by the binding of $^{125}$I-labeled ATF (S.R. and R. Yamamoto, unpublished data).

A peptide derived from the EGF-like domain of human urokinase, residues 12–32, with Cys-19 converted to Ala, competes with ATF for binding to uPAR with an $IC_{50}$ of 100 nM (30). To verify that bacteriophage displaying a uPAR ligand could be specifically selected by cell-surface uPAR, we constructed a positive control bacteriophage, encoding uPA 13-32C19A. As shown in Table 1, both COS-7 and baculovirus-infected Sf9 cells displaying human uPAR selectively enriched for the uPA 13-32C19A bacteriophage over a control bacteriophage by 500- and 800-fold, respectively. In addition, control cells expressing the substance P receptor did not enrich for this uPA bacteriophage. The random peptide bacteriophage display library, consisting of $10^7$ different 15-mers, was then affinity selected for three rounds alternately on Sf9 cells and COS-7 cells expressing uPAR. Enrichment for uPAR ligands was initially assessed by bacteriophage yield. After two rounds of selection the yield had increased 30-fold over the first round, and the third round showed a further increase of 130-fold to 5.4%, approximately that seen for the positive control bacteriophage. The overall yield increase was 4000-fold.

Individual plaques were picked from the third round eluate and subjected to DNA sequence analysis. From 66 plaques, 19 different DNA and peptide sequences were obtained, and these individual bacteriophage were tested for binding to cells displaying uPAR. Each of them showed 20- to 500-fold greater yields than an irrelevant bacteriophage. Peptides corresponding to the selected sequences were synthesized, purified, and tested as competitors in a uPAR binding assay with iodinated ATF as ligand (27). These results are summarized in Table 2, in comparison with known uPAR ligands.

To further map the sites of receptor interaction for these ligands, we asked whether the clone 20 synthetic peptide blocks uPAR binding of bacteriophage displaying three peptides: uPA 13-32C19A, clone 16, and the homologous clone 20. The results, shown in Fig. 1, indicate that clone 20 peptide prevents >95% of the binding of all three bacteriophage to uPAR, whereas a control peptide had little effect.

Table 1. Affinity selection of a positive control bacteriophage displaying human urokinase 13-32C19A by cell-surface uPARs

| Cells/receptor | % recovery: Positive control | % recovery: M13 | Ratio positive control/M13 |
|---|---|---|---|
| Sf9/uPAR | 6.8 | 0.009 | 756 |
| Sf9/SPR | 0.008 | 0.01 | 0.8 |
| COS-7/uPAR | 1.5 | 0.003 | 500 |
| COS-7/ETRB | 0.006 | 0.003 | 2.0 |
| COS-7/Mock | 0.003 | 0.003 | 1.0 |

Sf9 cells ($1 \times 10^6$ cells) expressing full-length human uPARs or substance P receptors (SPR) were used 48 h postinfection with the appropriate recombinant baculoviruses. COS-7 cells expressing uPAR or the human endothelin B receptor (ETRB) were tested 48 h posttransfection with a DEAE-dextran chloroquine protocol essentially as described (26) at $2 \times 10^5$ cells per well in six-well tissue culture dishes. Controls consisted of mock-transfected COS cells and polyhedron mutant baculovirus (CΔ3)-infected Sf9 cells. uPAR expression was validated by using murine monoclonal antibody 3936 (American Diagnostica) and anti-mouse horseradish peroxidase conjugate with tetramethylbenzidine as substrate (31). In each experiment, between $6 \times 10^8$ and $1.2 \times 10^9$ plaque-forming units of positive control bacteriophage and a 10-fold excess of M13 were used as input. Bacteriophage were selected as described for selection from the 15-mer library. Elutions were with 0.5 ml of 6 M urea (pH 3) for 15 min at room temperature with gentle mixing. The eluted bacteriophage were separated from the cells by centrifugation and then neutralized with 10 μl of 2 M Tris base. The inputs and eluates were titered with JM103 cells on 5-bromo-4-chloro-3-indolyl β-D-galactoside plates containing 1 mM isopropyl β-D-thiogalactopyranoside to distinguish between M13 plaques (blue) and positive control phage plaques (clear).

## DISCUSSION

The molecular details of the interaction between uPA and uPAR have been investigated by several groups. Stoppelli *et al.* (32) showed that the N-terminal fragment of uPA (residues

Table 2. Receptor binding affinities and sequences of peptides derived from panning of 15-mer phage library on human uPAR

| Clone | Sequence | Frequency* | $IC_{50}$, μM† |
|---|---|---|---|
| 20 | AEPMPHSLNFSQYLWYT | 11 | 0.01 |
| 26 | AEHTYSSLWDTYSPLAF | 8 | 0.34 |
| 54 | AELDLWMRHYPLSFSNR | 1 | 0.38 |
| 16 | AESSLWTRYAWPSMPSY | 5 | 0.40 |
| 12 | AEWHPGLSFGSYLWSKT | 6 | 0.40 |
| 18 | AEPALLNWSFFFNPGLH | 1 | 1.0 |
| 9 | AEWSFYNLHLPEPQTIF | 2 | 1.0 |
| 11 | AEPLDLWSLYSLPPLAM | 2 | 2.0 |
| 42 | AEPTLWQLYQFPLRLSG | 1 | 2.5 |
| 48 | AEISFSELMWLRSTPAF | 1 | 5.0 |
| 75 | AELSEADLWTTWFGMGS | 1 | 7.0 |
| 17 | AESSLWRIFSPSALMMS | 1 | 8.0 |
| 13 | AESLPTLTSILWGKESV | 1 | 8.0 |
| 10 | AETLFMDLWHDKHILLT | 4 | 8.0 |
| 44 | AEILNFPLWHEPLWSTE | 2 | 9.0 |
| 14 | AESQTGTLNTLFWNTLR | 8 | 10.0 |
| 38 | AEIKTDEKMGLWDLYSM | 1 | 23.0 |
| 19 | AEMHRSLWEWYVPNQSA | 9 | >23 |
| 36 | AESHIKSLLDSSTWFLP | 1 | >47 |
| — | uPA 1–135 (ATF) | — | 0.00012 |
| — | uPA 12–32C19A | — | 0.25 |

Phage were selected as described in the text. Peptide sequences were determined by translation of DNA sequencing results from single-stranded phage templates. Receptor binding assays were done in 96-well microtiter plates as described.

*Number of separate times a given DNA and amino acid sequence was obtained from randomly picked plaques.

†Apparent inhibition constant of the synthetic peptide or uPA fragment for the uPAR–ATF interaction.

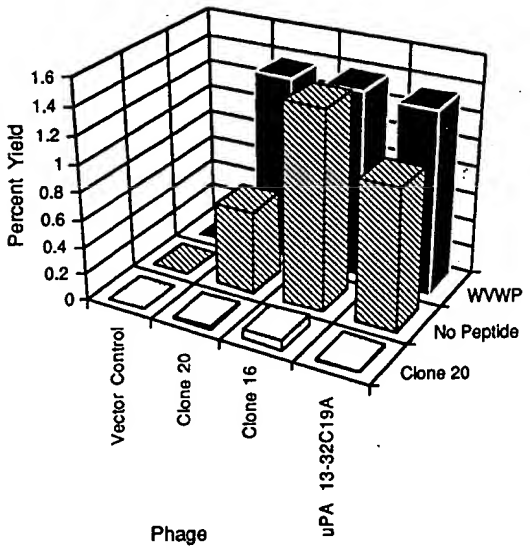


FIG. 1. Soluble peptide competition for phage binding to immobilized uPAR. Bacteriophage displaying uPA 13-32C19A (Table 1), clone 16, clone 20 (Table 2), or no peptide (LP67) ($6 \times 10^8$–$2 \times 10^9$ plaque-forming units input) were incubated in uPAR-coated wells, with and without the indicated peptides, washed, and eluted as described. The peptides used as competitors at 2 $\mu$M final concentration were clone 20 or a control peptide (WVWP). The data are shown as percentage yield of the various input phage in the presence and absence of the indicated peptides; the standard errors of these measurements are in the range of 20–50%.

1–135 including the EGF-like and kringle domains) bound to U937 cell uPAR with equal or greater affinity than uPA itself, thus separating the catalytic and receptor binding regions. Subsequent work showed that deletion of the EGF-like domain (residues 9–45) from pro-uPA reduced uPAR binding at least $10^3$-fold (33) and that recombinant human uPA EGF-like domain (residues 1–45 or 1–48) binds to uPAR with affinity comparable to that of uPA (ref. 34; S.R. and J. Stratton-Thomas, unpublished data).

Appella *et al.* (30) used limited proteolysis and synthetic peptides to further localize the key receptor binding region of uPA. They showed that a disulfide-bonded cyclic peptide encompassing residues 12–32 of uPA, with Cys-19 converted to Ala, competed with labeled ATF for binding, with an $IC_{50}$ of 40 nM. Cleavage of ATF at Lys-23 or Phe-25 drastically reduced uPAR binding, further supporting the requirement for a specific ligand secondary structure (30). The structures of molecules related to the uPA EGF-like domain, including human EGF, murine EGF, and human transforming growth factor $\alpha$, have been determined by multidimensional NMR methods (35, 36). In these cases the central loop of the molecule, corresponding approximately to residues 13–32 in the uPA EGF-like domain, is the major secondary structural element, composed of two $\beta$ strands and a turn. Preliminary NMR analysis of the uPA EGF-like domain shows a similar secondary structure (E. K. Bradley, personal communication).

uPAR consists of three domains of ≈90 amino acids, which are related to the Ly-6 superfamily (25). The murine and human receptors show little or no cross-species binding (37). A chymotryptic fragment of human uPAR (residues 1–87) can be cross-linked to ATF (38), and recent work with a closely related receptor fragment (residues 1–92) suggests that the determinants of species specificity reside primarily in the first 13 residues, as determined with chimeric receptors (39).

We have used a 15-mer random peptide library displayed on bacteriophage M13 to isolate uPAR ligands. This library consists of $10^7$ different bacteriophage displaying three to five copies of a random 15-mer peptide near the N terminus of the P3 protein in the sequence $AEX_{15}P_6$ (15). Since the total number of 15-mer peptides is $3.3 \times 10^{19}$, only 1 in $10^{12}$ sequences has been displayed. The probability of a particular sequence being expressed is not uniform due to codon bias, so that some sequences will be further underrepresented (40, 41). Despite these limitations, we have identified 15-mer peptide ligands, the most potent of which (clone 20, $IC_{50}$ = 10 nM) is only 25- to 100-fold less potent than uPA itself (32, 42). The less potent peptides likely represent suboptimal sequences due to the limited size of the library. None of the selected peptides contained cysteine residues, despite a requirement for correct disulfide bond formation in high-affinity uPA binding to uPAR, as only a single disulfide-bonded isomer of recombinant uPA EGF-like domain (residues 1–48) binds human uPAR (S.R. and J. Stratton-Thomas, unpublished data).

An alignment of the more potent uPAR binding peptides ($IC_{50} < 5$ $\mu$M), based on conserved subsequences, is shown in Fig. 2. It is difficult to align all of the bacteriophage-derived sequences with each other, and especially with the receptor binding region of uPA, since the bacteriophage peptides are linear and the uPA binding region is cyclic. There also appear to be two subsets of phage-derived peptides, with the motifs FXXYLW (clones 20 and 12) and LWXXY (clones 16 and 26). These common sequence motifs are in different registers within the variable region (Fig. 2), suggesting that subsequences of the 15-mers are likely active.

The vast majority of the peptides identified compete for ATF binding, consistent with the idea that they bind to uPAR in the uPA binding site. This suggests either that the bacteriophage peptides are capable of a relatively defined secondary structure, mimicking a disulfide-bonded loop, or that the molecular details of the binding interactions of uPAR with the bacteriophage-derived peptides and uPA are overlapping but distinct. Alternatively, these different ligands may bind to the same uPAR amino acid residues, as seen for the two unrelated binding sites on human growth hormone binding to the human growth hormone receptor (43). The likelihood of a common set of molecular interactions between uPAR and both uPA and clone 20 peptide is strengthened by the observation that the peptide is a species-specific ligand for

| Sequence Name | Alignment | IC50 (uM) |
|---|---|---|
| | Set 1 | |
| 20 | AEPMPHSLNFSQYLWYT | 0.01 |
| 12 | AEWHPGLSFGSYLWSKT | 0.40 |
| 48 | AEISFSELMWLRSIPAF | 5.0 |
| | Set 2 | |
| 26 | AEHTYSSLWDTYSPLAF | 0.34 |
| 54 | AELDILWMRHYPLSFSNR | 0.38 |
| 16 | AESSLWIRYAWPSMPSY | 0.40 |
| 18 | AEPALLNWSFFFNPGLH | 1.0 |
| 9 | AEWSFYNLHLPEPQITF | 1.0 |
| 11 | AEPLDIWSLYSLPPLAM | 2.0 |
| 42 | AEPTLWQLYQFPLRLSG | 2.5 |

FIG. 2. Alignment of highest-affinity bacteriophage-derived peptide sequences. The highest-affinity peptide sequences derived from the bacteriophage display library selection are aligned. The sequences are divided into two subsets, which have subsequence motifs of FXXYLW and LWXXAr (Ar = Y, F, H, or W). Residues in boldface are conserved within or between sequence subsets. A speculative alignment of these sequences with each other is shown.

uPAR and does not bind to the murine uPAR (H. Y. Min and S.R., unpublished data).

The frequency of a given bacteriophage sequence in the third round pool of bacteriophage does not correlate with the inhibition constants for ATF binding (see, for example, clones 14 and 20 in Table 2). This could be due to variable protease sensitivity of the displayed peptides or to the polyvalent display method used (44). Alternatively, some of the bacteriophage may bind to sites other than the uPA ligand binding site. The possibility that other functional binding sites are present on uPAR is suggested by the three-domain structure of the molecule, in which the first domain is required for uPA binding, and the other two domains are of unknown function (38, 39).

In summary, we have identified a family of potent peptide antagonists for the human urokinase receptor from a random peptide bacteriophage library by selection on receptor-bearing cells. This work validates the bacteriophage peptide display technology using cell-surface receptors. These molecules will serve as leads for the discovery of pharmaceutical agents that inhibit cell-surface proteolysis and as unique tools for analysis of the uPA–uPAR interaction.

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# *Communication*

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# Identification and Characterization of Src SH3 Ligands from Phage-displayed Random Peptide Libraries*

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**We have used the Src homology 3 (SH3) domain to screen two phage-displayed random peptide libraries, each containing $2 \times 10^8$ unique members, and have identified a series of high affinity peptide ligands. The peptides possess similar proline-rich regions, which yield a consensus Src SH3-binding motif of RPLPPLP. We have confirmed this motif by screening a phage-displayed peptide library biased for SH3 ligands and identifying the same consensus sequence. Binding studies using synthetic peptides suggest that the RPLPPLP motif is important for SH3 binding and confers specificity for the Src SH3 domain, and that residues which flank the motif may also contribute to binding. Peptides that contain the RPLPPLP motif compete Src, but not Abl or phospholipase Cγ, SH3 interactions with SH3-binding proteins from cell lysates ($IC_{50}$ = 1–5 μM). Furthermore, RPLPPLP-related peptides are able to accelerate progesterone-induced maturation of *Xenopus laevis* oocytes. A similar acceleration has been observed in oocytes treated with activated, but not normal, *Xenopus* Src, suggesting the possibility that the peptides are able to antagonize the negative regulation of Src activity by Src SH3 *in vivo*.**

Src represents a family of at least nine non-receptor protein-tyrosine kinases; members of this family share an overall structural organization comprising a series of catalytic and non-catalytic domains (1). The carboxyl-terminal half of Src contains the protein-tyrosine kinase catalytic domain and a negative regulatory tyrosine (Tyr-527), whose phosphorylation results in the inhibition of kinase activity (2). The amino-terminal half of Src contains two highly conserved non-catalytic regions termed Src homology (SH)[1] domains 2 and 3. SH2 and SH3 domains are composed of approximately 100 and 60 amino acids, respectively, are found in a variety of proteins with important roles in signal transduction, and have been shown to mediate critical protein-protein interactions in a number of signaling pathways (3). SH2 domains bind phosphotyrosine (Tyr(P)); residues that surround the Tyr(P) moiety determine SH2 specificity (4). SH3 domains recognize short proline-rich stretches of amino acids, although the basis for SH3 specificity remains unclear (5).

Many mutations that result in increased Src protein-tyrosine kinase and transforming activity map to the Src SH2 and SH3 domains (6–9), suggesting a negative regulatory role for these domains. There is evidence that both domains are involved in maintaining Tyr(P)-527-mediated inhibition of Src kinase activity (9–11). Whereas the discovery that SH2 domains bind Tyr(P)-containing sequences has established an explanation for the role of the SH2 domain in Tyr(P)-527-mediated inhibition of Src activity (12), the role of the SH3 domain in Src regulation remains unclear. Identification of Src SH3 ligands may provide insight into the role of the SH3 domain in regulating Src and may lead to the discovery of SH3-binding proteins critical for Src function and transforming activity.

Efforts to identify natural ligands for different SH3 domains have led to the characterization of a number of SH3-binding proteins, including the Abl SH3-binding proteins 3BP1 and 3BP2 (13); the Ras GTP-exchange factor SOS, which interacts with Grb2 (14, 15); p85 phosphatidylinositol 3′-kinase, which binds Src, Fyn, and Lck SH3 (16, 30); dynamin, which binds SH3 domains from Grb2, Src, Fyn, Fgr, and phospholipase Cγ (17); and the Src SH3-binding protein AFAP-110 (18). These proteins possess short proline-rich segments, some of which have been directly implicated in SH3 binding. Although a number of SH3 ligand consensus motifs have been proposed (13, 17), factors that govern the affinity and specificity of SH3 interactions remain poorly defined. Attempts to define SH3-binding consensus motifs and to elucidate rules for ligand specificity have been complicated by the small number of SH3 ligands characterized to date, the often low degree of similarity between ligands for the same SH3 domain, and the presence of multiple overlapping putative SH3-binding regions within the same protein.

Screening complex random peptide libraries has proved to be a powerful strategy for identifying peptide ligands for a variety of target molecules. Such libraries have been used to identify peptide epitopes for monoclonal (19) and polyclonal (20) antibodies, as well as ligands for a variety of proteins, including streptavidin (21, 22), calmodulin[2] (24), and the endoplasmic reticulum protein BiP (23).

To expand upon the limited repertoire of known SH3 ligands and to gain a more sophisticated understanding of the role of the SH3 domain in Src function, we have used the Src SH3 domain to isolate a series of peptide ligands from phage-displayed random peptide libraries. The peptides possess similar proline-rich regions, which yield a consensus sequence of RPLPPLP. Binding studies using synthetic peptides suggest

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[1] The abbreviations used are: SH, Src homology; Tyr(P), phosphotyrosine; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; RIPA, radioimmune precipitation buffer.

[2] B. K. Kay, N. B. Adey, H. L. Hanson, I. D. Clark, J. D. Brennan, and A. G. Szabo, manuscript in preparation.



EXHIBIT C

that the RPLPPLP motif is important for SH3 binding and confers specificity for the Src SH3 domain, and that residues which flank the motif may also contribute to binding. Furthermore, RPLPPLP-related peptides act as potent and specific antagonists of Src SH3 interactions with SH3-binding proteins from cell lysates and accelerate progesterone-induced maturation of *Xenopus laevis* oocytes.

## EXPERIMENTAL PROCEDURES

*Preparation of GST-SH3 Fusion Proteins*—PCR fragments encoding full-length Grb2 or c-Src SH3 (amino acids 87–143) were cloned into the *Bam*HI site of pGEX-2T. pGEX-derived constructs expressing GST fusion proteins containing the SH3 domains of Yes, Crk, Abl, and phospholipase Cγ were obtained from M. Sudol (Rockefeller University), M. Matsuda (NIH, Tokyo, Japan), A. M. Pendergast (Duke University), and S. Earp (University of North Carolina), respectively. Fusion proteins were prepared as described previously (26). Microtiter wells were coated with 5–20 μg of GST-SH3 fusion protein in PBS; blocked with 100 mM $Na_2HCO_3$, 1% bovine serum albumin; and washed with five applications of PBS, 0.1% Tween 20, 0.1% bovine serum albumin (Buffer A). The amount of protein bound to each well was quantified by anti-GST antibody-based ELISA (Pharmacia Biotech Inc.) or with a GST-binding peptide.[3]

*Isolation and Characterization of Src SH3-binding Phage*—Library screens were performed as described previously (20). Briefly, $5 \times 10^{11}$ plaque-forming units of T9 (20), T12,[2] or T13[4] library in Buffer A were incubated in GST-Src SH3-coated wells for 2 h. The wells were washed five times with buffer A, and bound phage were eluted with 50 mM glycine-HCl (pH 2.2). Recovered phage were amplified in DH5αF′ *Escherichia coli* and affinity-purified twice more, as above. Binding phage were plated to yield isolated plaques, from which phage stocks and DNA were produced for binding experiments and dideoxy sequencing (27), respectively. Binding was confirmed by applying equal amounts of phage to wells coated with GST-Src SH3 or GST, washing the wells with Buffer A, and detecting bound phage by anti-phage antibody-based ELISA (Pharmacia Biotech Inc.).

*In Vitro Peptide Binding Assays*—Biotinylated peptides (Research Genetics, Birmingham, AL; Chiron Mimotopes, Victoria, Australia; Cytogen Corp., Princeton, NJ) were synthesized with a GSGS peptide linker between the biotin moiety and the $NH_2$ terminus of the indicated sequence. Peptide purity was assessed by high pressure liquid chromatography and/or mass spectrometry. Binding experiments were performed as above, except for the use of 1 μM peptide instead of phage. Bound peptide was detected by streptavidin-alkaline phosphatase-based ELISA (Sigma).

*Peptide Competition of GST-SH3 Affinity Precipitations of Cell Lysates*—NIH 3T3 cells were labeled overnight in Dulbecco's modified minimal medium, 10% dialyzed fetal calf serum, 80 μCi/ml Tran$^{35}$S-label (ICN, Irvine, CA). Labeled cells were washed with PBS, lysed in RIPA buffer, and clarified as described (28). Lysate from $1.5 \times 10^6$ cells was incubated with 10 μg of glutathione-agarose-immobilized GST-SH3 fusion protein ± peptide in a final volume of 250 μl. Pelleted beads were washed with 1 ml each of RIPA; RIPA, 1% deoxycholate, 0.1% SDS; and PBS, then resuspended in SDS-polyacrylamide gel electrophoresis sample buffer, boiled, and subjected to SDS-polyacrylamide gel electrophoresis (7.5%). Labeled proteins were detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

*Progesterone-induced Oocyte Maturation*—Stage VI oocytes were prepared and injected as described previously (29). Oocytes were injected with 40 nl of 100 μM peptide, 1 mg/ml monoclonal antibody 327, or water. After injection, oocytes were incubated in 2 μg/ml progesterone (Sigma) and were scored at hourly time points for germinal vesicle breakdown.

## RESULTS AND DISCUSSION

In an effort to identify peptide ligands for the Src SH3 domain, we undertook the screening of three different phage-displayed peptide libraries. Two of the libraries, T9 and T12, each contain $2 \times 10^8$ unique clones expressing 22- or 36-amino acid-long random peptides, respectively, fused to the amino terminus of M13 protein III. As such, they represent a vast source of molecular diversity and have the potential to encode

**A**

| | | |
|---|---|---|
| T9.2 | SSFDQQDWDYSIAEKMHPIRPGF | RELPPLP PSRASFGGGASRPSR |
| T9.4 | SSSGYVVPKRLGDMREYNAHPGLHVPPN | SPLPPLP THLQSSRPSR |
| T9.6 | SSRGEGNNIISSRPFLSNSDPGVSNKLTGRW | GPLPPLP NDSRPSR |
| T12.6 | STAPWGLRVAHEGGVLK | RPLPIPP VTRPSR |
| T12.4 | STNVWVTGSVIARGAQS | RPLPIPP ETRPSR |
| T12.3 | STNDVDWMHMWNSGGPH | RRLPPTP ATRPSR |
| T9.5 | SSDNWARRVHASELIYTDLSPGILLAQ | RQLPPTP GRDPSHSRPSR |
| T9.3 | SSYNDLGTRPVSEVIKYDYFPGYSQHVITPDGSYST | RPLPSRP SR |
| T12.2 | STRWSHSWPGYVGGANPSPAT | RPLPTRP SR |
| T12.5 | STAHSLWDWGTFSGVSHK | SRLPPLP TRPSR |
| T9.7 | PGYARIVSYRP | RALPSPP SASRPSR |
| T9.1 | SSESPLMYNRVGALQSLTSVPGSMHHFALQ | RRLPRTP PPASRPSR |
| T12.7 | STAVSFRFMPGGGGAFYST | RPVPPIT RPSR |
| T12.1 | STMYGVSWLSSGSGG | ILAPPVP PRNTRPSR |
| Consensus | | RPLPPLP |

**B**

| | |
|---|---|
| T13.5 | STP RPLPMLP TTRP |
| T13.6 | ST RPLPILP RTTRP |
| T13.1 | ST RPLPSLP ITTRP |
| T13.7 | STS RPLPSLP TTRP |
| T13.3 | STN RPLPMIP TTRP |
| T13.10 | STP RPLPLIP TTRP |
| T13.8 | ST RPLPPTP LTTRP |
| T13.2 | ST RSLPPLP PTTRP |
| T13.9 | ST RPQPPPP ITTRP |
| T13.4 | ST RQLPIPP TTTRP |
| Consensus | RPLPPLP |

FIG. 1. **Deduced amino acid sequence of peptides displayed by Src SH3-selected phage.** *A*, sequence of peptides isolated from T9 and T12 random peptide libraries. *B*, sequence of peptides isolated from T13 biased peptide library. Residues within the peptides that match the consensus have been aligned. *Bold* residues are fixed in all library clones; sequences included in synthetic peptides are *underlined*.

virtually every possible 7-amino acid-long peptide. The third library, T13, contains approximately $1 \times 10^6$ unique members expressing 8-amino acid-long peptides encoded by the DNA sequence $((C/A)NN)_8$. This encoding scheme results in a biased peptide library wherein the constituent amino acids are represented in the following proportions: 6R:4P:4L:4T:3I:2H:2Q:2K:2N:2S:1M. Many of these residues have been identified in naturally occurring SH3-binding sequences (13–17, 30).

Phage clones expressing SH3-binding peptides were affinity-purified with immobilized GST-Src SH3 fusion protein. After three rounds of purification, isolated clones were confirmed as binding to Src SH3 (data not shown). Fig. 1 lists the amino acid sequences of peptides expressed on Src SH3-binding phage; similar sequences within the peptides have been aligned and yield a consensus motif of RPLPPLP. Peptides derived from screening either the random peptide libraries (expressing all 20 residues at each position, Fig. 1*A*) or the biased peptide library (expressing 11 residues at each position, Fig. 1*B*) yield the same consensus motif. These data suggest that the sequence RPLPPLP represents at least a significant part of the preferred Src SH3-binding motif. Support for this conclusion comes from a recent study in which a synthetic peptide library biased for SH3 ligands (of the form *XXXPPXPXX*) was used to identify Src SH3-binding peptides with the consensus motif RXLPPLPR (31). Our identification of the RPLPPLP motif from two random peptide libraries with no *a priori* bias for any particular sequence characteristic, together with the identification of the same motif from two different biased peptide libraries, provides strong evidence that the sequence RPLPPLP represents at least a significant part of the preferred Src SH3-binding motif.

Biotinylated peptides corresponding to sequences displayed by Src SH3-binding phage were synthesized and assayed for direct binding to immobilized GST-Src SH3 fusion protein (Fig. 2*A*). Each of the library-derived peptides bound GST-Src SH3 over GST background. This binding was not simply a consequence of the proline-rich nature of the library-derived peptides, since a proline-rich peptide from the cytoskeletal protein

[3] A. B. Sparks and B. K. Kay, unpublished observations.
[4] N. B. Adey, A. B. Sparks, and B. K. Kay, unpublished observations.

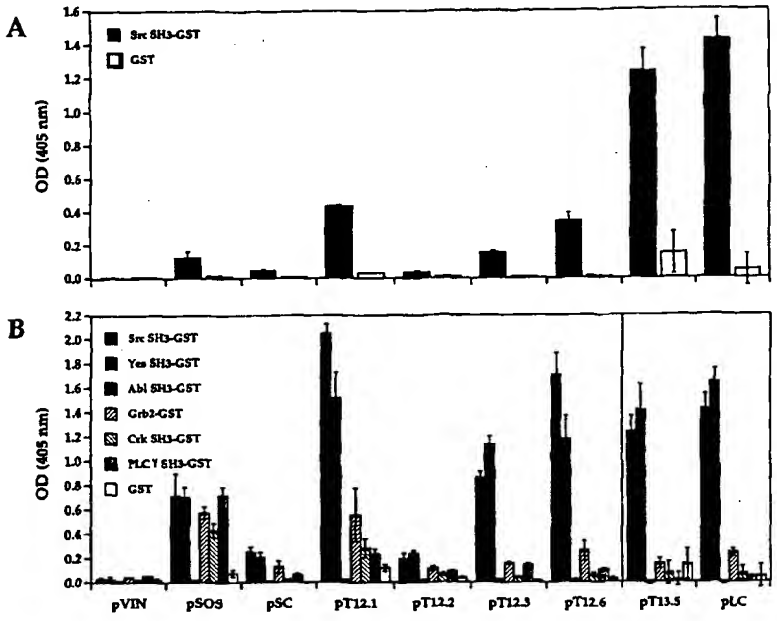


FIG. 2. **Binding strength and specificity of synthetic Src SH3-binding peptides.** *A*, relative binding of peptides to GST-Src SH3. ELISA signal after 30 min. *B*, relative binding of peptides to various GST-SH3 fusion proteins. ELISA signal after 30 min (peptides pT13.5 and pLC) and after 240 min (all other peptides). Peptides (see Fig. 1 for library-derived sequences; other sequences are as follows: pVIN, LAPPKPPLPEGEV; pSOS, GTVEPVPPPVPPRRRPESA; pSC, RPLPPPLP; pLC, STPRPLPPLPTTR) were bound to equal amounts of immobilized GST-SH3 fusion protein. Bound biotinylated peptide was detected with streptavidin-alkaline phosphatase ELISA. Each point was performed in triplicate; values are average absorbance at 405 nm above bovine serum albumin background ± S.D.

vinculin (pVIN) exhibited no binding. Furthermore, all but one of the library-derived peptides bound Src SH3-GST better than did a previously characterized SH3-binding peptide from SOS (pSOS) (14). We observed a strong correlation between the peptides' similarity to the RPLPPLP motif and their affinity for Src SH3; experiments comparing the relative binding of various phage clones produced equivalent results (data not shown). Moreover, while none of the clones expressed the sequence RPLPPLP, a peptide (pLC) consisting of the RPLPPLP motif flanked by residues encoded by clone T13.5 bound Src SH3 better than all other peptides tested. Taken together, these data provide further evidence that RPLPPLP represents at least a significant part of the preferred Src SH3-binding motif.

The single exception to the correlation between RPLPPLP similarity and Src SH3 binding, pT12.1, bound well despite its lack of similarity to the RPLPPLP motif (Fig. 2*A*). As pT12.1 exhibits other unique characteristics (see below), it may represent a distinct solution to Src SH3 binding. Interestingly, pT12.1 resembles SH3-binding sequences in SOS and a set of Src SH3 ligands identified by Yu *et al.* (31) that did not match their consensus motif.

Whereas pLC produced the strongest Src SH3-binding signal, a peptide consisting of the RPLPPLP motif alone (pSC) bound poorly (Fig. 2*A*). Moreover, SH3-binding motifs tend to cluster near one end of the phage-displayed random peptides, adjacent to sequences that are fixed in every clone (Fig. 1*A*). This tendency has not been observed in binding populations selected with other proteins (20) and suggests that fixed flanking residues may facilitate SH3 binding by the phage. Thus, although RPLPPLP represents at least a significant part of the preferred Src SH3-binding motif, sequences that flank RPLPPLP may contribute to SH3 binding. This contribution may be an indirect consequence of increased stabilization of the RPLPPLP binding conformation or a direct consequence of additional SH3-contact residues. The use of second generation random peptide libraries that fix all or part of the RPLPPLP motif and randomize flanking residues may help define additional residues important for Src SH3 binding.

To evaluate the specificities of the library-derived peptides, we determined their relative binding to SH3 domains from Src, Yes, Grb2, Crk, Abl, and phospholipase Cγ (Fig. 2*B*). Of the peptides that demonstrated significant Src and Yes SH3 binding, only pT12.1 and pSOS bound appreciably to SH3 domains from Grb2, Crk, and phospholipase Cγ. Thus, peptides related to the RPLPPLP consensus motif exhibit specificity for the Src SH3 domain (and the highly similar Yes SH3 domain), whereas the more divergent peptides pT12.1 and pSOS recognize a broad range of SH3 domains. That none of the peptides recognize Abl SH3 is consistent with the fact that they do not conform to a previously defined Abl SH3 consensus motif (13) and provides further evidence that SH3 domains possess distinct ligand specificities.

GST-SH3 fusion proteins may be used to affinity precipitate SH3-binding proteins from cell lysates. We therefore tested the ability of library-derived peptides to compete with cellular proteins for binding to the Src SH3 domain. As shown in Fig. 3, recovery of SH3-binding proteins with GST-Src SH3 could be blocked in a dose-dependent fashion ($IC_{50}$ = 1 to 5 μM by PhosphorImager densitometry) by pT12.1, pT12.6, and pLC, although 100 μM pVIN had no effect. Similar results were obtained in experiments using GST-Yes SH3 (data not shown). This competition was specific for Src SH3, since none of the peptides blocked association with Abl SH3, and only pT12.1 significantly blocked binding to phospholipase Cγ SH3. These results are consistent with binding data presented in Fig. 2. Thus, the RPLPPLP-related peptides are able to specifically compete Src SH3 interactions with natural SH3-binding proteins *in vitro*, suggesting their potential for disrupting SH3-mediated events *in vivo*.

*X. laevis* oocytes injected with mRNA encoding constitutively active Src undergo progesterone-induced maturation at an accelerated rate relative to oocytes injected with water or c-Src mRNA (32). Since the Src SH3 domain is thought to be involved in the negative regulation of Src kinase and transforming activity (6–11), we have examined the effect of Src SH3-binding peptides upon oocyte maturation. Fig. 4 shows that maturation was accelerated by approximately 1 h in oocytes injected with pT12.6 or pLC, but not in oocytes injected with water, pVIN, the Src SH3-specific monoclonal antibody 327, or pT12.1. The magnitude of this effect is similar to that seen with injection of mRNA encoding constitutively active Src (32). Interestingly, only RPLPPLP-related peptides with specificity for Src SH3 were competent to accelerate maturation. Although the peptides may be exerting their effect via a target other than the Src

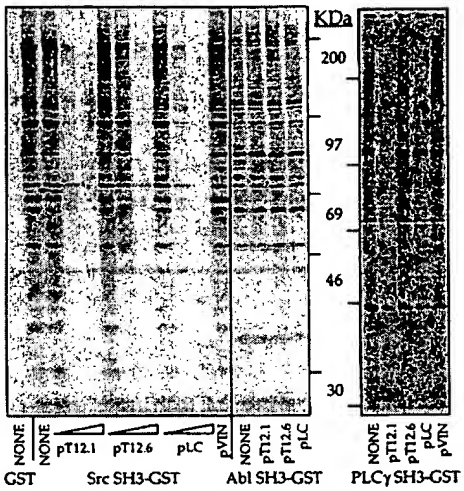


FIG. 3. **Peptide competition of Src SH3-GST precipitation of proteins from cell lysates.** $^{35}$S-Labeled NIH 3T3 cell lysates were incubated with 1 μM glutathione-agarose-coupled GST-SH3 fusion protein ± peptide. Final peptide concentrations were 1, 10, and 100 μM for the series indicated by ◿ (otherwise 100 μM).

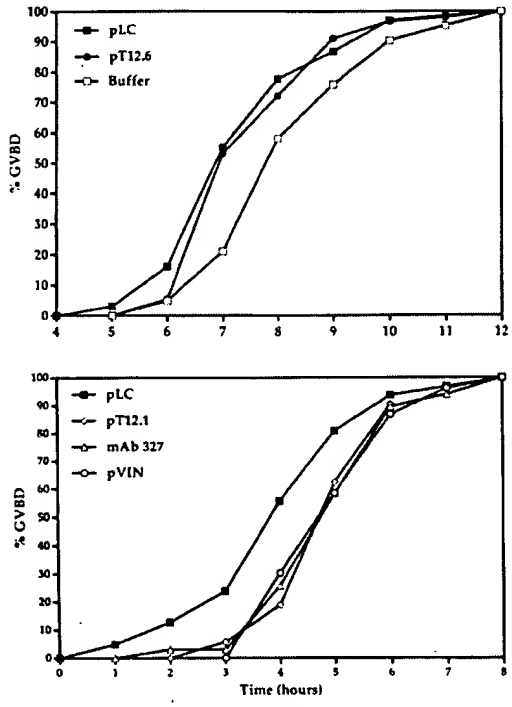


FIG. 4. **Peptide acceleration of progesterone-induced oocyte maturation.** Stage VI oocytes were injected with peptide, monoclonal antibody 327, or injection buffer. For each treatment, 100 oocytes were injected, treated with progesterone, and scored for germinal vesicle breakdown (*GVBD*) at hourly time points. Results are expressed as a percentage of oocytes that underwent germinal vesicle breakdown by the indicated time.

SH3 domain, these results suggest the possibility that the RPLPPLP-related peptides are able to antagonize the negative regulation of Src activity by Src SH3 *in vivo*. Future studies examining the effect of these peptides upon Src kinase activity, substrate phosphorylation, and protein-protein interactions may clarify the specific mechanism whereby the peptides exert their effect and may contribute to our understanding of the role of the Src SH3 domain *in vivo*.

We have used phage-displayed peptide libraries to identify the Src SH3-binding motif RPLPPLP. Because this motif was identified from libraries with no *a priori* bias for any specific sequence characteristics, we conclude that RPLPPLP represents at least a significant part of the preferred Src SH3-binding sequence. Second generation biased libraries may provide a means of extending the Src SH3-binding motif, as well as addressing the binding specificities of other SH3 domains. The isolation of biologically active Src SH3 ligands from libraries incorporating no information regarding natural SH3-binding sequences suggests that non-biased peptide libraries may represent a general and efficient means of discovering peptide ligands for, and characterizing binding specificities of, other domains thought to be involved in protein-protein interactions, such as pleckstrin homology domains (33) and armadillo repeats (25), even when no information is available regarding their natural ligands.

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# CD28 and CTLA-4 Have Opposing Effects on the Response of T cells to Stimulation

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**Summary**

The importance of the B7/CD28/CTLA-4 molecules has been established in studies of antigen-presenting cell–derived B7 and its interaction with the T cell costimulatory molecule CD28. CTLA-4, a T cell surface glycoprotein that is related to CD28, can also interact with B7-1 and B7-2. However, less is known about the function of CTLA-4, which is expressed at highest levels after activation. We have generated an antibody to CTLA-4 to investigate the consequences of engagement of this molecule in a carefully defined system using highly purified T cells. We show here that the presence of low levels of B7-2 on freshly explanted T cells can partially inhibit T cell proliferation, and this inhibition is mediated by interactions with CTLA-4. Cross-linking of CTLA-4 together with the TCR and CD28 strongly inhibits proliferation and IL-2 secretion by T cells. Finally, results show that CD28 and CTLA-4 deliver opposing signals that appear to be integrated by the T cell in determining the response to activation. These data strongly suggest that the outcome of T cell antigen receptor stimulation is regulated by CD28 costimulatory signals, as well as inhibitory signals derived from CTLA-4.

Recent work has demonstrated that CD28, a protein expressed on resting and activated cells, is the major costimulatory molecule for proliferation of T cells (1–3). CD28 engagement via antibodies augments the proliferation of T cells in response to immobilized anti-TCR antibodies (4). Additionally, antibody engagement can supply costimulation to T cells encountering APCs deficient in costimulation (4, 5) and prevents the resultant anergic state that otherwise occurs in long-term clones (4). Finally, Fab fragments of anti-CD28 can fully block proliferation by costimulation competent APCs (4).

Several lines of evidence indicate that B7-1 (CD80) and B7-2 (CD86) (6) are the major costimulatory ligands on the APC. First, a chimeric fusion protein of CD28 binds B7-1 and B7-2 (7, 8). Second, anti-B7 antibodies block T cell activation by a variety of APCs (9, 10). Finally, induction of expression of B7-1 or B7-2 by transfection with cDNAs confers costimulatory activity on cells that do not otherwise provide costimulation (11–14). Interestingly, APCs and especially dendritic cells, which are thought to be involved in the early phases of T cells activation, express moderate levels of functional B7-2 without activation (6, 15). These levels increase nearly 100-fold with overnight activation, enhancing their APC function.

B7-1 and B7-2 also bind CTLA-4, a close relative of CD28. Chimeric fusion proteins consisting of the ectodomain of CTLA-4 bind B7-1 and B7-2 (8, 16–18) and can block T cell activation by costimulation competent accessory cells (10, 12). Notably, studies with soluble fusion protein indicate that CTLA-4 binds both B7 family members with an affinity ~20-fold higher than that of CD28. This higher affinity probably accounts for the ability of the CTLA-4 Ig fusion protein to block costimulation in vitro (10, 12) and to suppress graft rejection and antibody production in vivo (19, 20).

While the ability of CTLA-4 fusion proteins to bind CD28 ligands and block T cell activation is clear, the function of the native molecule has been obscure. Originally identified as cDNA cloned from a subtracted CTL clone library (21), CTLA-4 is homologous to CD28, especially in the extracellular domain, and both contain a conserved sequence motif, MYPPY, thought to be involved in B7 binding (22). Recent work has shown that CTLA-4 mRNA is expressed within a few hours of activation (23). Studies with mAbs to both human and mouse CTLA-4 demonstrated surface expression within 48 h of activation. However, functional studies have led to different conclusions about its role in activation. Linsley et al. in a study of human T cells found that anti–CTLA-4 antibodies enhanced proliferation of T cells activated with anti-CD3 and anti-CD28, suggesting that the function of CTLA-4 was to augment or sustain costimulation (24, 25). Walunas et al. found that both intact and monovalent fragments of antibodies to mouse CTLA-4 enhanced T cell responses in allogeneic MLR, but that intact antibody inhibited proliferation under conditions where Fc receptor cross-linking was provided (26). These results suggest that CTLA-4 might play a role in negative regulation of T cell activation.

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We describe here an analysis of CD28 and CTLA-4 signaling on highly purified T cells, noting the presence of B7 on the T cells themselves. The results indicate that the two molecules have opposing effects on lymphokine production and proliferation, and that the outcome of T cell activation is determined by integration of signals transduced by these two molecules.

## Materials and Methods

*Immunization and Hybridoma Production.* 6-wk-old golden Syrian hamsters received five footpad injections of 50 μl (packed volume) heat-killed *Staphylococcus* A bacteria coated with ~100 μg CTLA-4Ig (27) and suspended in 0.2 ml PBS. 3 d after the final injection, draining lymph nodes were removed, and lymphocytes were isolated and fused with the P3X3.Ag8.653 myeloma line using a standard polyethylene glycol fusion technique (28). Hybridoma supernatants were tested for reactivity to CTLA-4 Ig and for a lack of reactivity to CD4 Ig by ELISA (29). Hybridomas from positive wells were repetitively cloned by limiting dilution in the presence of irradiated mouse thymocyte feeder layers. Antibody 9H10 was specific for CTLA-4 by three criteria: (*a*) Reactivity against CTLA-4 Ig but not CD4 Ig; (*b*) the ability to block CTLA-4 Ig binding to B7 transfectants; (*c*) the ability to stain activated T cells but not freshly isolated T cells; and (*d*) the ability to stain a CTLA-4 transfectant but not control transfectants.

*Antibodies.* Antibodies used include anti-CD3 clone 500A2 (30), anti-CD28 clone 37.51 (31), anti-B7-1 clone 1610A (9), anti-B7-2 (17), anti-Vγ3 clone 536 (32), anti-class II MHC clone 28-16-8s (33), and anti-$IA^{d/b}$ clone BP107 (34). Conjugates of these antibodies were prepared in our laboratory. PE, biotin, and FITC conjugates of anti-CD4 and anti-CD8 were purchased from CALTAG Laboratories (South San Francisco, CA) and PharMingen (San Diego, CA).

*T Cell Activation Cultures.* Spleens from 4–6-wk-old BALB/c mice were harvested and minced, and suspensions were treated with Geys RBC lysis solution (35). Cells were cultured in RPMI containing 10% FCS and soluble anti-CD3 antibody at 10 μg/ml.

*Flow Cytometry.* $2 \times 10^5$ cells were suspended in 10 μl ice-cold PBS/1% calf serum/0.05% sodium azide. Antibodies were added for 30 min followed by two 4-ml washes in PBS/calf serum/sodium azide. Data were acquired on a FACScan® (Becton Dickinson and Co. (Mountain View, CA) and the LYSIS II program was used to electronically gate on relevant populations.

*Proliferation Assays.* LN cells were isolated from 6–8-wk-old BALB/c mice (Charles River Laboratories, Wilmington, MA). Isolated lymphocytes were obtained by mincing and filtration through nylon sieves. Cells were then treated with anti–class II antibodies 28.16.8s and BP107 and a mixture of rabbit and guinea pig complement (Accurate Chemical and Scientific Corp., Westbury, NY). Viable cells were isolated over lympholyte 1.119 (Sigma Chemical Co., St. Louis, MO) and residual Ig-positive cells were removed by repetitive panning on rabbit anti–mouse IgG coated tissue culture plates. Typical preparations analyzed by FACS® were typically found to be 99% $Thy1.2^+$ with <0.5% B220-positive cells. Round-bottomed 96-well plates were used for all assays. Where indicated, wells were coated with anti-CD3 at 0.1 μg/ml in 50-μl vol for 2 h at 37°C, then washed extensively and blocked for 30 min at 37°C with complete RPMI 1640. T cells were added at $10^5$ per well in 200 μl complete RPMI 1640. For soluble assays, anti-CD28 was added at a 1:1,000 dilution of ascites, anti–CTLA-4 was added at 10 μg/ml, and B7 antibodies were added at 2.5 μg/ml and 10 μg/ml for anti–B7-1 and anti–B7-2, respectively. For cross-linking assays, anti-CD8 was added at 4 μg/ml, anti-CD3 was added at 5 μg/ml, anti–CTLA-4 was added at 20 μg/ml, and anti-Vγ3 control antibody F536 was added to produce a constant total antibody concentration of 30 μg/ml. Polyclonal goat anti–hamster antibody (Pierce Chemical Co., Rockford, IL) was added at a final concentration of 20 μg/ml. All cultures were incubated at 37°C for 72 h, then pulsed with 1 μCi of [$^3$H]thymidine for an additional 16 h before harvesting.

*Antibody Incorporation onto Latex Microspheres.* Sulfate polystyrene latex microspheres of 5 ± 0.1 μM mean diameter were obtained from Interfacial Dynamics Corporation (Portland, OR). Approximately $1 \times 10^7$ beads per ml were suspended in PBS and the indicated antibodies were incubated for 1.5 h at 37°C. Anti-CD3 was added at 1 μg/ml to deliver the antigen-specific signal, and binding solutions were normalized with control antibody 536 to maintain a constant total antibody concentration of 5 μg/ml during binding. Previous data have shown maximal binding is achieved when this concentration is added per $10^7$ particles (36). Beads were washed extensively with PBS and resuspended in 1 ml RPMI–10% FCS and allowed to block for at least 30 min at room temperature. $10^5$ cells were incubated in 96-well cultures with $10^5$ each of the indicated bead preparation.

## Results

*Activation Induces Expression of CTLA-4 on Both $CD4^+$ and $CD8^+$ T cells.* Antibody 9H10 was used to assess CTLA-4 expression on freshly isolated and activated T cells. As shown in Fig. 1 *A*, CTLA-4 was undetectable on freshly isolated T cells. CTLA-4 was readily detected on T cells 48 h after stimulation by addition of anti-CD3 to splenocytes, and it was returned to resting levels by 72 h. CD28 expression was not greatly altered by stimulation. CTLA-4 was expressed by both $CD4^+$ and $CD8^+$ T cells, with significantly higher levels on the latter.

*Blockade of CTLA-4 or B7-1/2 Enhances Anti-CD28 Costimulation.* To determine the functional consequences of CTLA-4 engagement, soluble anti–CTLA-4 or anti-CD28 antibodies were added singly or together to cultures of purified T cells exposed to immobilized anti-CD3. As shown in Fig. 2, while anti-CD28 greatly enhances the minimal proliferation induced by CD3 stimulation, anti–CTLA-4 has no effect. This suggests that CTLA-4 does not function as an alternate costimulatory receptor. Despite its lack of costimulatory activity on its own, anti–CTLA-4 markedly increases T cell proliferation when given together with anti-CD28.

At least two mechanisms could account for the potentiating effects of anti–CTLA-4: enhancement of activation signals or removal of inhibitory signals. Given the observation that anti–CTLA-4 lacked costimulatory activity on its own, we favored the latter possibility—that blockade of CTLA-4 by the antibody might prevent interaction of CTLA-4 on the T cell surface with B7-1 and/or B7-2 and thus prevent delivery of an inhibitory signal. This is further suggested by the observation that anti–CTLA-4 Fab fragments have similar effects as anti–CTLA-4 mAbs in this assay system (data not shown). Since the cells used in our experiments were >99% $Thy1^+$ and contained no detectable MHC class-$II^+$ or $B220^+$ cells,

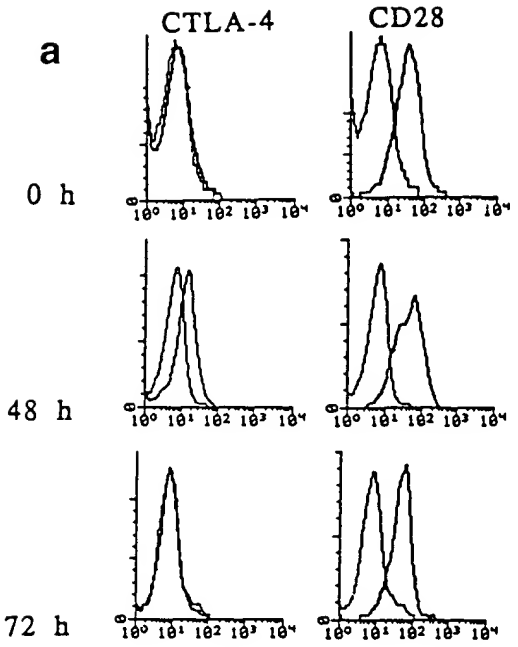


**Figure 1.** Expression of CTLA-4 and CD28 on resting and activated splenic T cells. BALB/c splenic cell suspensions ($2 \times 10^5$/ml) were stimulated in vitro with 10 μg/ml soluble anti-CD3. (*a*) Cells were double stained with Thy1.2PE and either hamster Ig-control FITC, anti-CTLA-4 FITC, or anti-CD28 FITC. Data were electronically gated for Thy1.2-positive cells; CTLA-4 and CD28 expressions are shown on freshly explanted cells and after 48- and 72-h incubations with anti-CD3. (*b*) 48-h cultures were stained for anti-CD4 biotin or anti-CD8 biotin followed by avidin tricolor and FITCylated irrelevant or CTLA-4 antibodies. Subpopulation-gated data show modestly higher CTLA-4 expression on CD8 populations.

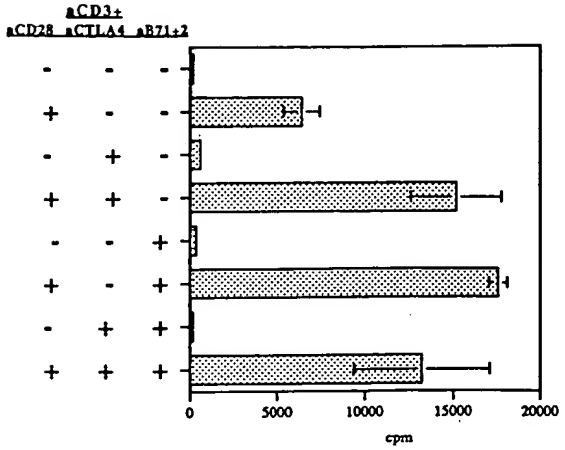


**Figure 2.** Influence of anti-CTLA-4 and anti-B7 antibodies on proliferative responses of purified lymph node T cells. $10^5$ BALB/c LN T cells were cultured for 72 h in 96-well microwells coated with 0.1 μg/ml anti-CD3. Anti-CD28 was used at a 1:1,000 dilution of ascites and B7 antibodies were used at 2.5 and 10 μg/ml for anti-B7-1 and anti-B7-2, respectively. Anti-CTLA-4 antibody 9H10 was added at 10 μg/ml.

this possibility would require that the T cells themselves provide a source of ligand. As shown in Fig. 3, flow cytometric analysis revealed that the freshly isolated T cells did indeed express significant levels of B7-2 and trace levels of B7-1.

To determine the functional consequence of B7 expression by T cells in our assay system, we determined the effects of anti-B7 antibodies on CD28-mediated costimulation. As shown in Fig. 2, anti-B7 antibodies by themselves had no significant effect on anti-CD3-induced T cell proliferation. The addition of anti-B7 antibodies to cultures containing anti-CD28 resulted in a threefold increase in proliferation over that obtained with anti-CD28 alone. A similar increase in proliferation was obtained when chimeric CTLA-4 Ig instead of anti-B7 antibodies was added to block B7 interactions (data not shown). The magnitude of the increase was similar to that obtained when anti-CTLA-4 is added to CD28-treated cells in the absence of B7 blockade. The addition of anti-CTLA-4 to cultures in which anti-B7 antibodies are present results in no further increase in CD28 costimulation; indeed, a slight but reproducible decrease is observed.

Together, these results suggest that T cells express B7 at levels that are insufficient to provide costimulation via CD28 engagement in the assay system used. However, perhaps because of the fact that CTLA-4 has a much higher affinity than CD28 for B7 binding, these levels are sufficient to generate a signal that at least partially inhibits activation. Blockade of the CTLA-4/B7 interaction with either anti-CTLA-4 or anti-B7 antibodies removes the inhibitory signal, resulting in an increase of the costimulatory effect of CD28 ligation.

*Cross-linking of CTLA-4 with the TCR and CD28 Inhibits T Cell Proliferation and IL-2 Production.* The results shown

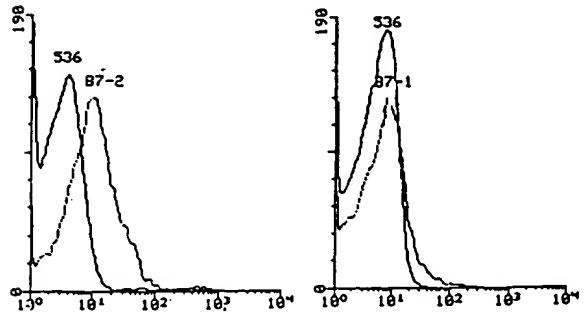


**Figure 3.** Expression of B7-2 and B7-1 on purified, freshly isolated lymph node T cells. Cells that were >99% Thy1.2$^+$ were isolated and stained with GL1 (anti-B7-2), 1610A (anti-B7-1), or irrelevant antibodies.

in Fig. 2 suggested that soluble, bivalent anti-CTLA-4 antibody was effective in blocking B7-mediated signals, but was inefficient in providing signals. We next examined the effects of using anti-hamster Ig to cross-link CD3, CD28, and CTLA-4 singly or together. As shown in Fig. 4, no proliferation was obtained when CD3, CD28, or CTLA-4 were cross-linked individually. As expected, cross-linking of CD3 together with CD28 resulted in potent costimulation, while cross-linking of CD3 and CTLA-4 had no effect. Co-cross-linking of CTLA-4 together with CD3 and CD28 consistently resulted in a 5- to 10-fold reduction in proliferation. This inhibition was largely reversed by the addition of IL-2 to the cultures, suggesting that the effect is not caused by toxicity. Finally, cross-linking of CTLA-4 with CD3 and CD28 also resulted in a profound decrease in IL-2 production in the cultures (Fig. 4 *B*). These results demonstrate that CTLA-4 can deliver signals that inhibit T cell responses to TCR ligation, and that the effects observed in the experiment presented in Fig. 2 most likely result from perturbation of B7/CTLA-4 interactions.

*CD28 and CTLA-4 Deliver Quantitatively Opposing Signals.* The preceding data indicate that CTLA-4 cross-linking in the presence of CD28 signaling can inhibit IL-2 secretion and proliferation. We next sought to determine whether signaling above the threshold for CTLA-4 inhibition is independent of the magnitude of CD28 costimulation, or whether the threshold increases as CD28 signaling increases. To address this issue, T cells were stimulated by incubation with polystyrene beads coated with a constant amount of anti-CD3 and varying amounts of anti-CD28 and anti-CTLA-4. As shown in Fig. 5, costimulation with increasing amounts of anti-CD28 in the absence of anti-CTLA-4 resulted in a gradual increase in proliferation, reaching at the highest dose a 1,500-fold increase over that obtained with anti-CD3 alone. The addition of increasing amounts of anti-CTLA-4 reduced that proliferation in a stepwise manner at all doses of anti-CD28. These results suggest that T cells integrate signals from CD28 with signals from CTLA-4, and the balance of these signals regulates the magnitude of the response to TCR ligation.

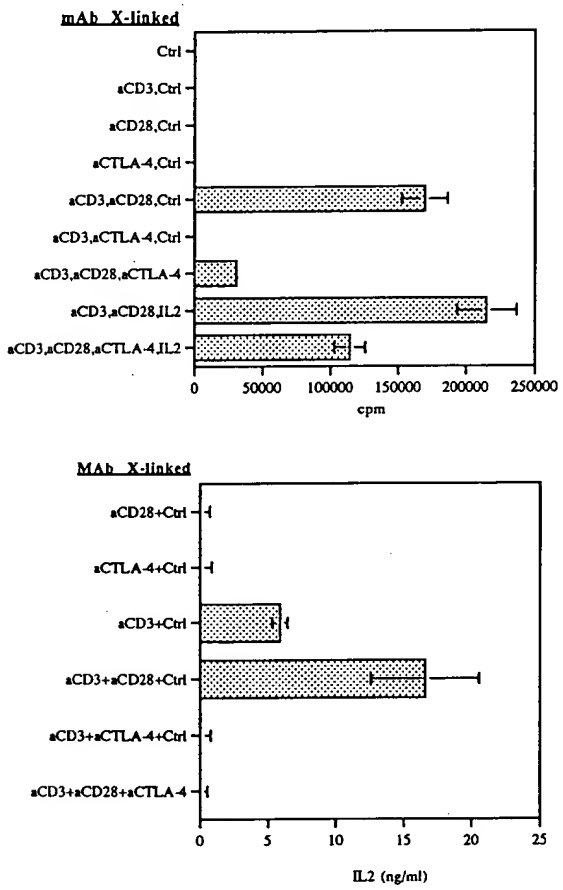


**Figure 4.** Cross-linked anti-CTLA-4 can diminish both proliferation and lymphokine production by purified LN T cells. $10^5$ BALB/c LN T cells were cultured with the indicated hamster antibodies together with control hamster antibodies. Anti-hamster Ig antibody was added at 20 μg/ml to cross-link. Where indicated, anti-CD3 was added at 5 μg/ml, anti-CD28 was added at 4 μg/ml, anti-CTLA-4 was added at 20 μg/ml, and control was added to normalize antibody concentration at 30 μg/ml. (*a*) Cells were cultured for 72 h, pulsed with 1 μCi [$^3$H]thymidine, and harvested after an additional 16 h. (*b*) Supernatants were removed and analyzed for IL-2 production at 48 h using an ELISA detection system.

## Discussion

The results presented here clearly demonstrate that CTLA-4 does not serve as a functional alternative to CD28 in providing costimulatory signals to T cells. This finding is in agreement with earlier studies showing that CTLA-4 did not replace CD28 function in CD28 mutant mice (37). The finding that anti-CTLA-4 increases proliferation of T cells activated by anti-CD3 and anti-CD28 is in agreement with the results of Linsley et al. (24). However, the fact that a similar result is obtained when blocking antibodies to B7 are included suggests that this apparent cooperativity of CTLA-4 is in fact a result of removal of preexisting inhibitory B7-CTLA-4 in-

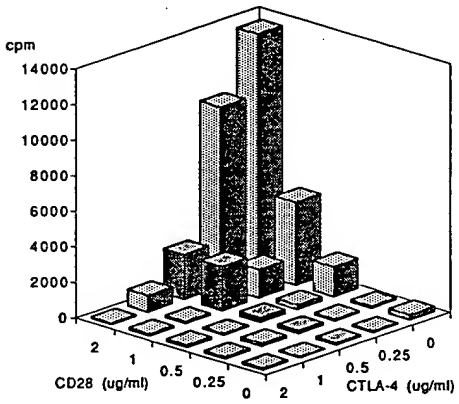


**Figure 5.** Proliferation in response to a constant CD3 signal is affected by the relative concentrations of CD28 and CTLA-4 signals. $10^7$ 5 μM microspheres were coated with 1 μg/ml of anti-CD3, the indicated concentrations of anti-CD28 and anti-CTLA-4, and control hamster Ig constituting a total antibody concentration of 5 μg/ml. $10^5$ coated spheres were incubated in 96-well cultures with $10^5$ purified LN T cells for 72 h, pulsed with 1 μCi [$^3$H]thymidine, and harvested after an additional 16 h.

teractions by the soluble CTLA-4 antibodies rather than a synergism between the two antibody-generated signals. The ability of CTLA-4 to directly signal is supported by the fact that cross-linking of anti–CTLA-4, either with second antibody or by presentation immobilized on beads, results in inhibition of both IL-2 production and proliferation. This direct demonstration of signaling by CTLA-4 supports the report of Walunas et al. (26) that CTLA-4 is a negative regulator of T cell activation.

Our demonstration of a dynamic competition between CD28 and CTLA-4 indicates that in addition to CD3 and CD28 signal integration, there exists an integration point for CTLA-4–derived signals. At present there is little knowledge of the biochemical events that follow CD28 or CTLA-4 ligation. There have been reports that CD28 stimulation results in induction of protein tyrosine kinase activity (38), and recent evidence suggest the Tec family kinase ITK represents one associated protein kinase (39). In addition, it has been demonstrated that phosphoinositides accumulate in T cells stimulated by ligation of CD28 with B7-1, suggesting an involvement of phosphatidylinositol 3′-kinase (PI3K)[1] with CD28 (40). In this regard, it is of interest that the cytoplasmic domains of both CD28 and CTLA-4 contain the sequence YM/VXM, a motif found in several growth factor receptors that associate with PI3K (41, 42). Several recent reports have documented a stimulation-induced association between CD28 and PI3K, and it has been reported that mutation of the PI3K-binding motif destroys the costimulatory activity of CD28 (43–47). These findings strongly suggest that binding of PI3K plays an important role in CD28 signaling. With respect to CTLA-4, however, there have been contradictory findings. Whereas a chimeric protein containing the cytoplasmic domain of CTLA-4 was unable to bind PI3K (46), another study reported the coprecipitation of PI3K activity with CTLA-4 (48). In any event, these findings raise the possibility that CD28 and CTLA-4 might compete for PI3K and affect its role in subsequent signal transduction.

It is also possible that CD28 and CTLA-4 signals might intersect at later stages in the pathway. It has been demonstrated that the CD28 and CD3 pathways intersect at the level of the MAP kinase JNK (49). CTLA-4 might in someway interfere with this coupling, thus preventing costimulation. Finally, it is possible that CTLA-4 signals might interfere with those of CD28 even further downstream by interfering with IL-2 transcriptions or mRNA stabilization (46, 50).

Our results further suggest that regulation of the outcome of T cell stimulation is a complex process with regard to events at the cell surface. It is clear that in the absence of costimulatory signals provided by the B7 family, T cells do not proliferate. It appears that even small amounts of B7, such as those present on T cells themselves, are ineffectual in supporting CD28-mediated costimulation of anti-CD3 responses. This appears to be less a consequence of the absence of CD28 signal being delivered, but rather a result of an inhibitory signal delivered through CTLA-4. This implies that either CTLA-4 is quickly expressed after activation and aborts the response, or that CTLA-4 is expressed at functionally significant levels on resting T cells. At higher levels of B7 expression, as might be encountered on activated dendritic cells and activated B cells, CTLA-4 expression on the T cells might become limiting, and the costimulation provided by CD28 becomes dominant. As expression of CTLA-4 rises after activation, the signals generated through CTLA-4 might become dominant and terminate the response. Decay of CTLA-4 expression with time would allow the T cell to return to a state where the CD28 costimulatory signal would predominate. In any event, accumulating evidence suggests that in addition to antigen receptor and CD28-mediated signals, a third signal, provided by CTLA-4, is important in determining the outcome of T cell activation.

[1] *Abbreviation used in this paper:* PI3K, phosphatidylinositol 3′-kinase.

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# CTLA4 mediates antigen-specific apoptosis of human T cells

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ABSTRACT **The regulation of T cell-mediated immune responses requires a balance between amplification and generation of effector function and subsequent selective termination by clonal deletion. Although apoptosis of previously activated T cells can be induced by signaling of the tumor necrosis factor receptor family, these molecules do not appear to regulate T-cell clonal deletion in an antigen-specific fashion. We demonstrate that cross-linking of the inducible T-cell surface molecule CTLA4 can mediate apoptosis of previously activated human T lymphocytes. This function appears to be antigen-restricted, since a concomitant signal T-cell receptor signal is required. Regulation of this pathway may provide a novel therapeutic strategy to delete antigen-specific activated T cells.**

Whereas events regulating the initiation and amplification of T cell-mediated immune responses are well characterized, the cellular interactions and signaling events resulting in antigen-specific peripheral T-cell clonal deletion are ill understood. Following clonal expansion and effector function, previously activated murine T cells are selectively depleted in the periphery (1–4). Several cell surface molecules, including Fas and tumor necrosis factor receptors, induce apoptosis of previously activated T cells (5–8). However, these molecules are not T-lineage-restricted, and there is no evidence that they delete T cells in an antigen-specific manner. The identification of a T-cell antigen-specific apoptotic signal or a T-cell surface-restricted molecule that might associate with apoptotic ligands would be central to our understanding of the regulation of T cell-mediated immunity.

The B7 family of costimulatory molecules delivers a critical costimulatory signal through CD28 on the T cell (9–11). Blockade of CD28 signaling is sufficient to induce anergy (12), and in the absence of interleukin 2 (IL-2), anergized T cells undergo apoptosis (13). In addition to CD28, B7 family members have an alternative T-cell surface receptor, CTLA4 (cytotoxic T lymphocyte-associated protein 4) (14). CTLA4 is T-cell-restricted, appears after T-cell activation (15), and shares 31% overall amino acid identity with CD28 (16). A striking feature of CTLA4 is the total phylogenetic conservation of the cytoplasmic domain (17). Although it has been proposed that CD28 and CTLA4 are functionally redundant, anti-CD28 Fab totally blocks T-cell responses to costimulation by both B7-1 and B7-2 (10, 11). Here we demonstrate that CTLA4 does not provide a redundant CD28-like signal. In contrast, crosslinking of CTLA4 of previously activated T cells results in antigen-specific apoptosis.

## MATERIALS AND METHODS

**Proliferation Assays and Cytokine Detection.** T-cell subsets were obtained from peripheral blood mononuclear cells (PBMC) by selective removal with monoclonal antibodies (mAbs) and immunomagnetic beads. T-cell blasts were isolated after culture in medium containing phytohemagglutinin (PHA) for different time periods. Alloreactive T-cell clones with specificity for HLA-DR7 were made from HLA-DR7-negative individuals (12). T-cell clones were activated by culture with an *HLA-DR7* homozygous lymphoblastoid line (LBL-DR7). T-cell clones selected did not express either B7-1 or B7-2 either before or after activation. Microtiter plates were coated with anti-CD3 mAb at a concentration of 1 μg/ml. T cells ($10^5$ cells per well) were cultured at 37°C for 72 hr in the presence of mAbs (10 μg/ml). Where indicated, IL-2 was added (final concentration, 100 units/ml). Thymidine incorporation during the last 16 hr was assessed as an index of mitogenic activity. IL-2 accumulation at 24 hr was assayed by ELISA.

**Transfectants.** Chinese hamster ovary (CHO) cells were transfected with human CD28 cDNA (CHO-CD28). Successful expression of CTLA4 was obtained after the extracellular domain of CTLA4 was PCR-amplified from human CTLA4 cDNA and ligated into pCDM8 vector containing the glycophosphatidylinositol anchor of human CD58 (18). CHO-CTLA4 cells were sorted and subcloned by B7-1-Ig fusion protein. COS cells were transfected with cDNA encoding human HLA-DR α chain (DRα) and HLA-DR7 β chain (together designated transfectant t-DR7) alone or coexpressing DR7 and human B7-1 (t-DR7/B7-1) or human B7-2 (t-DR7/B7-2). NIH 3T3 cells were transfected with cDNA encoding human DR7 and B7-1 (t-DR7/B7-1). Cells were fixed in paraformaldehyde (0.4%) prior to use.

**mAbs.** BALB/c mice were immunized with the recombinant extracellular domain of human CTLA4 or phorbol 12-myristate 13-acetate-activated human PBMC. Three fusions of murine spleen cells and Sp2/0 resulted in 18 cloned mAbs that reacted with recombinant CTLA4 by ELISA. Five of these mAbs were reactive with CHO-CTLA4 cells. Anti-CD28, anti-CD28 Fab, anti-B7-1, anti-B7-2 (B-70; PharMingen), BB1, CTLA4-Ig (13) or control Ig, or anti-CTLA4.1 Fab was added to appropriate cultures (final concentration, 10 μg/ml).

**Epitope Mapping.** Epitope mapping was performed by phage display (19).

**Binding Assays.** CTLA4-Ig was bound to plates, and either biotinylated B7-1-Ig or B7-2-Ig fusion protein was evaluated as a binding ligand by ELISA.

## RESULTS

**Anti-CTLA4 mAbs Do Not Costimulate T-Cell Proliferation or IL-2 Secretion.** We generated a panel of anti-CTLA4 mAbs to determine the function of this molecule on resting and previously activated peripheral blood human T cells and T-cell clones. Fig. 1*A* summarizes the binding of the five anti-CTLA4 mAbs and of a representative anti-CD28 mAb to CHO cells expressing cell surface CD28 or CTLA4. An anti-CD28 mAb

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Abbreviations: mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; IL-2, interleukin 2; PHA, phytohemagglutinin; DR7, HLA-DR7; t, transfectant; TCR, T-cell antigen receptor.

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**A**

| Antibody | Clone | Isotype | Expression by Flow cytometry | |
|---|---|---|---|---|
| | | | CHO-CD28 | CHO-CTLA4 |
| anti-CD28 | 3D10 | IgG2a | + | − |
| anti-CD28/CTLA4 | 7G11 | IgG2a | + | + |
| anti-CTLA4.1 | 4D3 | IgG2b | − | + |
| anti-CTLA4.2 | 3D6 | IgG1 | − | + |
| anti-CTLA4.3 | 3D8 | IgG2b | − | + |
| anti-CTLA4.4 | 4E3 | IgG2b | − | + |

**B**

mAb 7G11 Selected Phage Peptides

RYLT[PPTTL]SRPVSQNSART
RDVTLA[APFFI]GGPPATVHT
DEVSAASW[PPYYI]WERVPHA
LRPTHQFL[PAYYL]SNRQLSL
HFDYMIRNR[TPYYQ]WPTVGQ
RDRTGAVVGTQ[PPYWL]GAPR
GFWGMEHNLTTGLS[PTWYL]K
SWNLRSLPDQPIGSP[PPYWL]
FAFKLGGNGLGGATY[PPYPI]

hCTLA4 Peptide RP 454: KVELMYP[PPYYL]GIGNGTGG

**C**

| Antibody | CD4 + T cells + anti-CD3 | |
|---|---|---|
| | [$^3$H] TdR (cpm) | IL-2 (pg/ml) |
| media alone | 257 | <20 |
| anti-CD28 | 74,541 | 950 |
| anti-CD28/CTLA4 | 58,865 | 780 |
| anti-CTLA4.1 | 289 | <20 |
| anti-CTLA4.2 | 204 | <20 |
| anti-CTLA4.3 | 325 | <20 |
| anti-CTLA4.4 | 319 | <20 |
| anti-CD28 + anti-CTLA4.1 | 98,266 | 1020 |

mAbs 4D3, 3D6, and 3D8 and 4E3 Selected Phage Peptides

[LSWGLE]PWEGSFLWLTESPM
[LNWDID]SMPMGVYCDVPDSC
H[LSWGEE]VRHQGEPRADQPF
V[LTFLER]LLPAVVPRSCHPG
QS[LTFEDD]GSSFLIYRATSD
YQA[LTCFED]RSDCFFTSPFI
YTE[LTFAED]GLGSGKNLIPK
NKES[LSWADE]LVRKDPPHGV
PGLQS[LCWEED]AGLVPGQDS
TAERCVS[LTWNDD]TCDLTGA
RNWARRTSN[LSWDGD]DGSRG
YLANHFGWTS[MVWDAD]DTGH
VCALPDVGYEF[LTSHAD]EPC
GGLVMIERFNKLE[LTWADD]D

hCTLA4 Peptide RP 365: AATYMMGNE[LTFLDD]SIC

FIG. 1. Surface expression, epitope mapping, and function of anti-CTLA4 mAbs. (*A*) Isotype and surface expression of a panel of anti-CTLA4 mAbs. (*B*) Epitope mapping of the anti-CTLA4 mAbs. (*C*) Costimulation assessed by proliferation and IL-2 accumulation of CD4$^+$ T cells is provided by anti-CD28 mAb but not by anti-CTLA4 mAb. hCTLA4, human CTLA4; [$^3$H]TdR, tritiated thymidine incorporation.

(3D10) bound to CHO-CD28 but not CHO-CTLA4 cells. One mAb (7G11) bound to both *CD28*- and *CTLA4*-transfected CHO cells and is henceforth termed anti-CD28/CTLA4 mAb. Four mAbs bound only to CHO-CTLA4 and are termed CTLA4.1 (4D3), CTLA4.2 (3D6), CTLA4.3 (3D8), and CTLA4.4 (4E3). Epitope mapping of the anti-CTLA4 mAbs was performed by phage display and was confirmed with synthetic peptides (19). The epitopes recognized by the anti-CTLA4 mAbs were determined by using a phage display library expressing a random 20-mer amino acid segment and a micropanning protocol (19). Analysis of 20 randomly selected phage from a panning on the anti-CD28/CTLA4 mAb yielded the 9 unique peptide sequences shown in Fig. 1*B*. All of these sequences contained a core consensus sequence of five residues, Pro-Pro-Tyr-Tyr-(Leu or Ile, where Ile is isoleucine) within which aromatic residues phenylalanine and tryptophane were frequently substituted for tyrosine. The sequence Pro-Pro-Tyr-Tyr-Leu is present within the extracellular domain of CTLA4, within or very close to the CDR3 region, a portion of which is encompassed in synthetic peptide RP 454. The peptide RP 454 was used to confirm the epitope of anti-CD28/CTLA4 mAb and could compete with native CTLA4 for binding of anti-CD28/CTLA4 mAb (data not shown). The sequence identity of CD28 and CTLA4 across this region explains the cross-reactivity observed with this mAb on CHO-CD28 and CHO-CTLA4. The epitopes for mAbs CTLA4.1–CTLA4.4 were determined in separate pannings and were found to be similar for all four mAbs. Forty-two randomly selected phage obtained from panning on all four anti-CTLA4 mAbs yielded 14 unique peptide segments shown in Fig. 1*B*. From these peptides, the shared epitope was mapped to a stretch of six residues on CTLA4, Leu-Thr-Phe-Leu-Asp-Asp. All four mAbs recognized this core sequence and bound to peptide RP365 derived from the human CTLA4 sequence containing this motif. This shared epitope maps to the CDR2-like region. CTLA4.1 and CTLA4.2 mAbs immunoprecipitate a 41- to 43-kDa protein under nonreducing conditions from the cell surface of activated human peripheral blood T cells and from T-cell clones and immunoprecipitate human CTLA4 from *CTLA4*-transfected COS cells (data not shown). Western blotting with the anti-CD28/CTLA4 mAb confirms that the immunoprecipitated protein was CTLA4 and was not recognized by anti-CD28 mAb.

Since the anti-CTLA4 mAbs bound to two distinct antigenic regions on the CTLA4 molecule, we examined whether either of these regions bound B7 family members. CTLA4-Ig was bound to plates, and either biotinylated B7-1-Ig or B7-2-Ig fusion protein was evaluated as a binding ligand. Both B7-1-Ig and B7-2-Ig bound to CTLA4-Ig. However, neither anti-CD28/CTLA4 mAb nor anti-CTLA4.1 mAb could inhibit binding of either B7-1-Ig or B7-2-Ig (data not shown). Similarly, B7-1-Ig did not inhibit binding of anti-CTLA4.1 mAb or anti-CD28/CTLA4 mAb to CTLA4-Ig. Therefore, these antibodies do not bind to the B7-1 or B7-2 binding sites on CTLA4. This result was somewhat surprising since it had been suggested previously that the sequence Met-Tyr-Pro-Pro-Pro-Tyr was part of the binding site of both CD28 and CTLA4 to B7-1 (16).

**Anti-CTLA4 mAbs Decrease Proliferation and Induce Apoptosis of Previously Activated T-Cell Clones or T-Cell Blasts.** To determine whether one or more of these mAbs could deliver either a primary or CD28-like costimulatory signal, resting CD4$^+$ T cells were cultured with antibodies alone or in the presence of submitogenic doses of anti-CD3 mAb. None of these antibodies alone induced proliferation or IL-2 secretion by resting T cells (data not shown). Coculture of resting T cells with anti-CD3 mAb and either anti-CD28 or anti-CD28/CTLA4 mAb resulted in proliferation and IL-2 accumulation (Fig. 1*C*). In contrast, none of the anti-CTLA4-specific mAbs provided a CD28-like costimulatory signal, even when cross-linked by using a second-step antibody. However, the addition of anti-CTLA4 mAb over a wide variety of different concentrations provided an agonistic signal to that provided by anti-CD3 and anti-CD28 mAbs comparable to published results (15, 20). Similar patterns of results were observed whether the responding population was purified T cells or fractionated CD28$^+$ or CD8$^+$ cells (data not shown).

Since CTLA4 is expressed on activated but not resting T cells, we examined whether anti-CTLA4 mAb-mediated costimulation of previously activated T cells might induce proliferation and IL-2 production or, alternatively, might induce apoptosis. HLA-DR7-specific alloreactive T-cell clones were activated by culture for 24 hr with an irradiated *HLA-DR7*$^+$ homozygous lymphoblastoid cell line (LBL-DR7) that expresses both B7-1 and B7-2. Antigenic stimulation with LBL-DR7 markedly upregulated CTLA4 surface expression on the T-cell clones, but the activated clones do not express B7-1 or B7-2 (data not shown). Activated T-cell clones were rechallenged with NIH 3T3 cells transfected to express HLA-DR7 (t-DR7) either alone or in the presence of anti-CD28 or anti-CTLA4 mAbs. As seen in Fig. 2*A*, previously activated T-cell clones proliferated modestly in response to challenge with t-DR7 although no IL-2 accumulation was detected. The addition of anti-CD28 or anti-CD28/CTLA4 mAb resulted in significant proliferation and IL-2 accumulation. In contrast, the addition of anti-CTLA4.1 mAb did not stimulate proliferation but instead resulted in significantly decreased proliferation, no detectable IL-2 accumulation (Fig. 2*A*), and apoptosis (Fig. 3). Greater than 90% of the T-cell clones underwent apoptosis under these culture conditions as assessed by uptake of Hoechst 33342 dye (21). Since all four anti-CTLA4 mAbs shared a common epitope and had identical functional effects, the results in this and in all subsequent experiments are presented for anti-CTLA4.1 only. Apoptosis was also induced by the anti-CD28/CTLA4 mAb in the presence of anti-CD28 Fab fragments. Functionally this mAb costimulates by crosslinking CD28, resulting in IL-2 production that prevents CTLA4-mediated apoptosis. However, in the presence of anti-CD28 Fab fragments, this mAb crosslinks only CTLA4 and induces apoptosis. Crosslinking an independent epitope on CTLA4 suggests that it is highly unlikely that crosslinking the Leu-Thr-Phe-Leu-Asp-Asp region induces apoptosis purely by cross-reacting with another molecule. The addition of exogenous IL-2 to the wells containing activated T cells with t-DR7 and anti-CTLA4.1 mAb resulted in significant proliferation comparable to that observed in response to t-DR7 and prevented the induction of apoptosis.

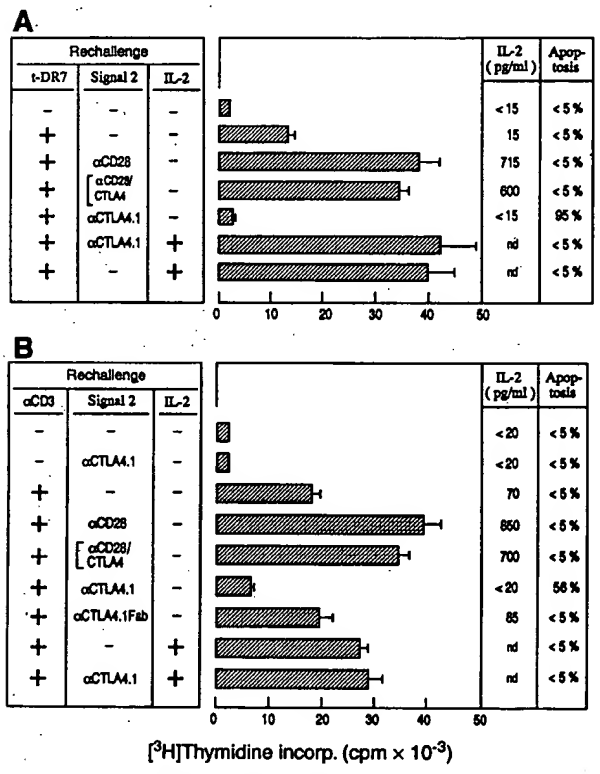


FIG. 2. Anti-CTLA4 mAbs induce decreased proliferation and apoptosis of previously activated T cells. (*A*) Previously activated alloreactive T-cell clones with specificity for HLA-DR7 were rechallenged with NIH 3T3 cells transfected with cDNA encoding human t-DR7 alone and with mAbs as shown under "Signal 2." The results are representative of six experiments on two T-cell clones. (*B*) CD4$^+$ T-cell blasts after culture of PBMC for 4 days in medium containing PHA. Thymidine incorporation (incorp.) was assessed during the last 16 hr of a 48-hr culture. IL-2 accumulation in the supernatant was assessed at 24 hr by ELISA. Apoptosis was assessed by the presence of DNA fragmentation and quantified by flow analysis of uptake of Hoechst 33342 and exclusion of propidium iodide. α, Anti-.

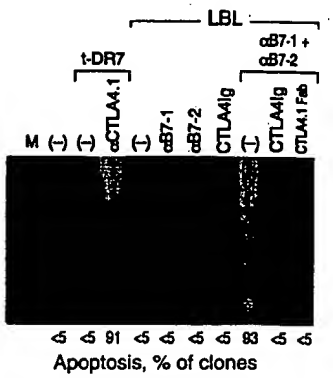


FIG. 3. Alloreactive T-cell clones undergo antigen-specific apoptosis after crosslinking with anti-CTLA4 mAb or a cell surface CTLA4-binding ligand on the surface of a lymphoblastoid cell line. Alloreactive T-cell clones were cultured for 8 hr in the absence (lanes "−") or presence of the indicated stimuli. Cells were washed in PBS and pelleted, DNA was extracted, and the samples were loaded into a 1% agarose gel containing 0.5 μg of ethidium bromide per ml and electrophoresed; DNA was visualized under UV light. Apoptosis was quantified by uptake of Hoechst 33342 and exclusion of propidium iodide. The results are representative of six experiments. Lane M, molecular weight markers (ϕX174 *Hae* III digest). LBL, lymphoblastoid cell line; α, anti-.

Following culture with either t-DR7 or anti-CTLA4.1 mAb for 24 hr, previously activated T-cell clones subsequently proliferated with the addition of exogenous IL-2. However, after culture for 24 hr with the combination of t-DR7 and anti-CTLA4.1 mAb, the T cells did not proliferate with the addition of exogenous IL-2, indicating that these cells had undergone antigen-specific clonal deletion (Table 1). Thus, a signal through the T-cell antigen receptor (TCR) in addition to CTLA4 crosslinking was required to induce apoptosis and clonal deletion.

Table 1. Antigen-specific clonal deletion

| Rechallenge conditions* | | | [3H]Thymidine incorporation,† |
|---|---|---|---|
| t-DR7 | Anti-CTLA4.1 | IL-2 | cpm |
| − | + | − | 26,388 ± 2,153 |
| + | − | − | 29,754 ± 1,736 |
| + | + | − | 1,370 ± 98 |
| + | + | + | 32,593 ± 2,074 |

Alloreactive T-cell clones were activated by culture with LBL-DR7 cells for 24 hr isolated on Percoll gradients, washed, and then rechallenged for 24 hr under the conditions shown. Cells were washed and cultured in medium containing 100 units of IL-2 per ml for 48 hr, and thymidine incorporation during the last 8 hr of culture was measured. The results are representative of four experiments on two T-cell clones.

*Prior to washing and 48-hr culture with IL-2.

†In last 8 hr of a 48-hr culture with IL-2.

To determine whether CTLA4 ligation could also induce apoptosis in previously activated normal human T cells, PBMC were cultured with phytohemagglutinin (PHA) for 4 days. $CD4^+$ T cell blasts were isolated and cocultured with anti-CD3 mAb in the presence of second signals as depicted in Fig. 2*B*. Anti-CTLA4 mAbs alone had no effect. Anti-CD3 alone induced modest proliferation of PHA-stimulated blasts accompanied by low levels of IL-2 accumulation. The addition of anti-CD28 or anti-CD28/CTLA4 mAbs enhanced both proliferation and IL-2 accumulation. Anti-CD3 in the presence of any of the anti-CTLA4 mAbs also resulted in decreased proliferation, absence of IL-2 accumulation (Fig. 2*B*), and apoptosis (data not shown). Apoptosis of >50% of the $CD4^+$ T-cell blasts was detectable as early as 8 hr of culture as assessed by uptake of Hoechst 33342 dye and flow cytometric analysis. Cross-linking of CTLA4 was necessary because anti-CTLA4.1 Fab did not decrease proliferation or induce apoptosis. The addition of exogenous IL-2 to anti-CTLA4.1 mAb protected against apoptosis. Similarly, the addition of anti-CD28 mAb also protected against apoptosis (data not shown). The apoptosis did not appear to be merely as a result of loss of production of IL-2 since $CD4^+$ PHA-stimulated blasts cultured in medium alone did not undergo apoptosis for periods up to 24 hr, by which time >80% of the cells cultured with anti-CD3 and anti-CTLA4.1 mAbs had undergone apoptosis. The apoptosis induced by CTLA4 crosslinking appeared to be specific, since anti-CD45, anti-CD45RA, anti-CD45RO, anti-CD4, anti-CD5, or anti-CD6 mAb crosslinking under identical culture conditions did not induce decreased proliferation or apoptosis in any of six experiments performed (data not shown). Similar levels of apoptosis were also observed in isolated $CD28^+$ (apoptosis observed in >70% by 8 hr) and $CD8^+$ (apoptosis in >60% by 8 hr) or unfractionated PHA-stimulated blasts (apoptosis observed in >60% by 8 hr) examined under the identical culture conditions (data not shown).

**B7-1 and B7-2 Do Not Induce CTLA4-Mediated Apoptosis.** Since CTLA4-mediated costimulation induced apoptosis of previously activated normal T cells and alloreactive T-cell clones, we sought to determine whether the known CTLA4-binding ligands mediated this function. Activated T-cell clones were rechallenged with COS cell transfectants t-DR7, t-DR7/B7-1, or t-DR7/B7-2. t-DR7 plus anti-CTLA4.1 suppressed proliferation of the T-cell clone and induced apoptosis (Fig. 4*A*). Both t-DR7/B7-1 and t-DR7/B7-2 augmented proliferation, and apoptosis was not observed. Addition of anti-CD28 Fab blocked the augmented proliferation induced by B7-1 and B7-2, demonstrating that the proliferative signal was indeed mediated via CD28. However, under these circumstances where binding to CD28 is blocked, binding of B7-1 or B7-2 to CTLA4 neither decreased proliferation nor induced apoptosis. Identical results were observed with PHA-activated human T cells (data not shown).

**An Alternative CTLA4 Ligand Induces Antigen-Specific Apoptosis of Previously Activated T Cells.** Since the two molecularly cloned CTLA4 ligands did not mediate antigen-specific clonal deletion, we attempted to determine whether alternative CTLA4 ligands might mediate this effect. Activated T-cell clones were rechallenged with LBL-DR7 cells alone or in the presence of mAb and fusion proteins as shown in Fig. 4*B*. mAbs were then added to block B7-mediated costimulation and IL-2 accumulation, since these ligands provided a positive signal that blocked anti-CTLA4 mAb-induced apoptosis. Blocking mAbs directed against B7-1, B7-2, and B7-3 (BB1) individually suppressed LBL-DR7-induced proliferation by up to 25%. Addition of CTLA4-Ig suppressed proliferation by 50% and totally blocked IL-2 accumulation, with the resulting proliferation comparable to that seen with t-DR7 alone. The addition of control Ig had no effect. In contrast, simultaneous

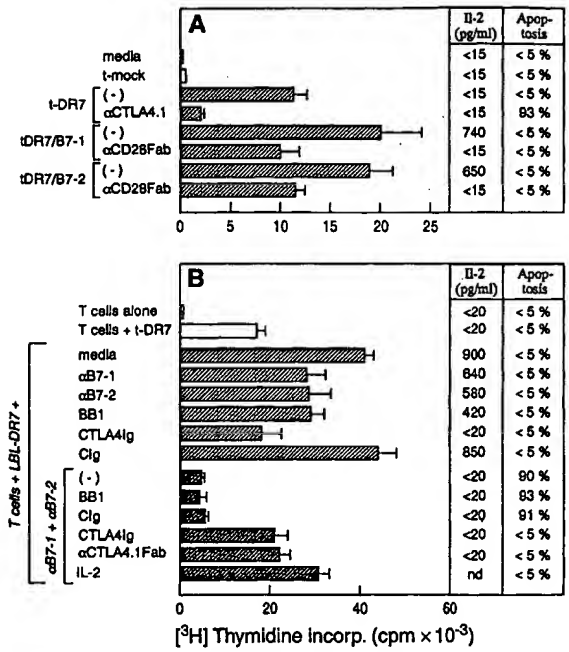


FIG. 4. CTLA4 crosslinking induces decreased proliferation and apoptosis of previously activated alloreactive T-cell clones. Thymidine incorporation (incorp.), IL-2 accumulation, and apoptosis are assessed as in Fig. 2. The results are representative of six experiments.

addition of anti-B7-1 and anti-B7-2 mAbs significantly suppressed proliferation and IL-2 accumulation (Fig. 4*B*) and, what is more important, induced apoptosis of >90% of the T-cell clones (Fig. 3). Further addition of BB1 mAb or control Ig to anti-B7-1 and anti-B7-2 mAbs had no effect on either proliferation or apoptosis. To determine whether this apoptosis was mediated via CTLA4, we added CTLA4-Ig or anti-CTLA4.1 Fab. Under either of these conditions, <5% of the T-cell clone underwent apoptosis (Fig. 3), and proliferation returned to levels comparable to that observed with t-DR7 alone (Fig. 4*B*). Apoptosis appeared to be antigen-specific, since LBL-DR1 cells under the identical culture condition did not decrease proliferation or induce apoptosis (data not shown). Again, the addition of IL-2 to anti-B7-1 and anti-B7-2 abrogated the suppression of proliferation and apoptosis. An identical pattern of results was again observed with PHA-stimulated blasts (data not shown). Whereas it is possible that cellular apoptosis occurs because of "lymphokine blockade," data presented suggests that this is not the case. T cells stimulated either with t-DR7 alone or with LBL-DR7 cells in the presence of CTLA4-Ig have no detectable IL-2 accumulation but do not undergo apoptosis. In addition, secondary challenge with medium alone does not induce apoptosis. Cellular death by apoptosis occurs in the T-cell clones only after 48 hr when cultured in medium alone, whereas CTLA4-mediated apoptosis is observed by 8 hr. CTLA4-Ig blocks both B7-1 and B7-2 yet does not have the same effect as the anti-B7-1 and anti-B7-2 mAbs. This suggests that it is not simply the absence of IL-2 production that results in apoptosis but rather binding to an alternative CTLA4 ligand that provides a positive apoptotic signal.

Taken together, these results demonstrate that a physiologic ligand for CTLA4 that mediates antigen-specific T-cell apoptosis: (*i*) is expressed on LBL-DR7 cells, (*ii*) is neither B7-1 nor B7-2, (*iii*) is a CTLA4-binding ligand because CTLA4-Ig blocked apoptosis, and (*iv*) signals through CTLA4 since anti-CTLA4.1 Fab also blocks apoptosis. These results are consistent with the hypothesis that CTLA4 provides not a redundant

CD28-like costimulatory pathway but rather a distinct signaling pathway capable, under appropriate conditions, of clonally deleting previously activated T cells.

## DISCUSSION

CTLA4 is a T cell-restricted cell surface molecule induced with TCR or CD28 activation. Its cytoplasmic tail is 100% conserved among chicken, mouse, and man, suggesting that this molecule is likely to mediate an important functional event. The present results show that, whereas CD28 costimulation by B7-1, B7-2, or anti-CD28 mAb induces secretion of multiple cytokines and upregulates IL-2 receptor expression on resting or previously activated T cells, under the identical conditions CTLA4 ligation provides no such stimulus. In contrast, concomitant antigen receptor signaling and CTLA4 crosslinking result in cellular apoptosis. Therefore, CTLA4 is not associated with a redundant CD28-like costimulatory pathway but rather a distinct signaling pathway capable of clonally deleting previously activated T cells when TCR signaling is not accompanied by significant IL-2 accumulation.

During an ongoing immune response, there is a balance between signals mediating activation and amplification and those that subsequently induce antigen-specific cellular deletion. Either cross-linking of CD28 or the common binding region of CD28/CTLA4 by mAbs or by their natural ligands B7-1 or B7-2 provides a positive costimulatory signal resulting in IL-2 accumulation. Our results suggest that signals that induce IL-2 accumulation are dominant, since they amplify the immune response and protect an ongoing immune response from CTLA4-mediated apoptosis. Since B7-1 and B7-2 are ligands for both CD28 and CTLA4, their function should be to mediate amplification rather than cellular depletion. During this interval of amplification, crosslinking of CTLA4 does not mediate apoptosis but, in contrast, can provide a weak synergistic costimulatory signal to CD28. After T-cell activation, CD28 engagement by B7-1 down-regulates CD28 synthesis and function as CTLA4 expression increases (22). Under conditions where the proliferative response is waning, crosslinking of CTLA4 in the absence of CD28-mediated costimulation can then induce cellular deletion of previously activated cells. The functional capacity to either costimulate or induce apoptosis depending on the state of activation of a cell is highly reminiscent of the functional repertoire of members of the tumor necrosis factor (TNF) receptor family (23). However, unlike the apoptosis induced by members of the TNF receptor family, CTLA4 crosslinking appears to mediate antigen-specific clonal deletion, since it requires a concomitant signal through the TCR. The mechanism whereby CD28-mediated signaling prevents apoptosis is unknown, but since this apoptosis can also be prevented by the addition of exogenous IL-2, it suggests that a viability signal is mediated through IL-2 receptor signaling.

Following activation of T-cell clones, at a time when CD28 expression is decreased and CTLA4 expression is maximal, neither t-DR7/B7-1 nor t-DR7/B7-2 induced apoptosis but rather provided a positive signal. Blocking B7-1 or B7-2 binding to CD28 with anti-CD28 Fab did not mimic the effect of crosslinking CTLA4 with CTLA4.1 mAb. These results suggest that neither B7-1 nor B7-2 induce CTLA4-mediated apoptosis. LBL-DR7 cells induce a secondary proliferative response of previously activated T-cell clones. The addition of anti-B7-1, anti-B7-2, or BB1 mAbs reduced proliferation and IL-2 production, but no apoptosis was observed. Similarly, CTLA4-Ig, which blocks all members of the B7 family, reduced proliferation and detectable IL-2 accumulation, but again no apoptosis was observed. In contrast, the identical LBL-DR7 cells in the presence of anti-B7-1 and anti-B7-2 mAbs inhibited proliferation and IL-2 production and, more importantly, induced >90% apoptosis of the T-cell clone. Subsequent blocking experiments demonstrate that the apoptotic ligand binds to CTLA4-Ig. Moreover, apoptosis is completely abrogated by a Fab fragment of the CTLA4.1 mAb, demonstrating that the natural ligand binds at or close to the same binding site as the CTLA4.1 mAb. Therefore, these results demonstrate that the natural ligand that can induce apoptosis is a CTLA4-binding molecule and binds to the identical site that the CTLA4.1 mAb bound on CTLA4.

There is no evidence that this ligand is a member of the B7 costimulatory family, but a potential candidate might be B7-3, a CTLA4-binding ligand that costimulates without inducing IL-2 accumulation (24). However, anti-B7-3 (BB1) mAb did not block CTLA4-mediated apoptosis. Regardless of the mechanism, the recognition of an antigen-specific pathway for clonal deletion of previously activated T cells would obviously have great potential for the termination of immune responses. Clinical manipulation of the balance between T-cell activation and subsequent clonal deletion, with particular relevance to autoimmunity and organ transplantation, should require both inhibition of positive signals leading to T-cell amplification and antigen-specific signaling with CTLA4 crosslinking.

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